The Characterisation and *In Vivo* Targeting of $G_{z\alpha}$.

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"The universe we observe has precisely the properties we should expect if there is, at bottom, no design, no purpose, no evil and no good, nothing but blind pitiless indifference.....

.....DNA neither cares nor knows. DNA just is. And we dance to its music"

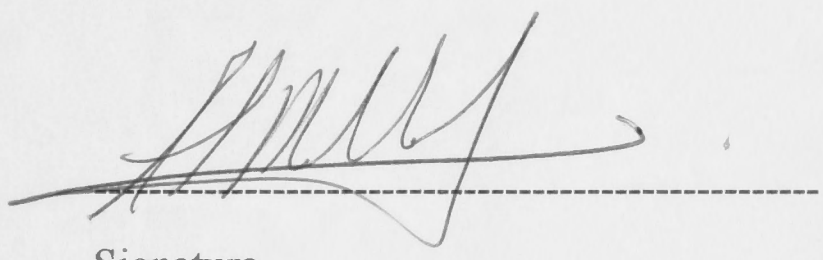
Richard Dawkins, River Out of Eden

In this quote taken from the recently published book, River out of Eden, Richard Dawkins makes it quite plain that although there may be nothing particularly special about DNA, it is after all the band master, and ultimate conductor of the destiny of life on this planet. Something to be remembered as we seek to understand the song, and even rewrite the melody. Various groups have sought to do this by reading each note in the sequence, and seeing how it may relate to its neighbours, through analogy with previously delineated sequences. Gene targeting technology allows us to see what happens to the melody after removing whole sections of the score. There are dangers to this approach, the change may be so subtle as not to be heard at all, or so disastrous that the whole performance is ruined. The trick is to choose the target carefully, and to remember the fallibility of the approach when making conclusions as to its success or otherwise.

This thesis describes the result of research undertaken within the John Curtin School of Medical Research, Australian National University, Canberra from 1/7/94 to 1/7/96.

The results and analysis presented in this thesis are my own original work accomplished under the supervision of Professor Ian A. Hendry, except where otherwise indicated in the text.

Albert S. Mellick



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Abstract.

G_z is a unique member of a class of heterotrimeric G-proteins which mediate cytosolic signalling pathways through a class of seven transmembrane spanning receptors. However, the *in vivo* function of G_z has been difficult to determine because the G_α guanine triphosphate binding subunit is resistant to both cholera and pertussis toxins. Localised to neuronal tissues, it has been speculated that $G_{z\alpha}$ may a wider play a role in mediating the signal generated by factors at the nerve terminal which do not themselves undergo retrograde axonal transport. To test this novel hypothesis and also determine which receptors couple/activate $G_{z\alpha}$ steps *in vivo* the decision was made to generate a $G_{z\alpha}$ null (or knockout) mouse. To this end a region of the mouse genome encompassing the first translated exon of mouse $G_{z\alpha}$ has been isolated, cloned, sequenced and used to stably disrupt the expression of $G_{z\alpha}$ in a C57Bl/6 mouse embryonic stem cell line *in vitro* which in turn have been used to produce animal chimæras. At this stage it is not known whether any of these mice are germline chimæras. Each are currently being interbred in the hope that this will in fact lead to the production of heterozygous knockout mice and later through the breeding of these heterozygous progeny, mice possessing both disrupted alleles of the $G_{z\alpha}$ gene.

Acknowledgments.

I would like to take the opportunity to thank a number of people for their invaluable assistance in completing this work:

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Abbreviations.

Adenosine Tri-Phosphate	ATP
Ammonium PerSulphate	APS
Annealing Time	AT
Annealing Temperature	T_A
Ammonium Acetate	NH_4OAc
Australian National University	ANU
Bluescript II SK/KS(+/-)	BSK/KS(+/-)
Brain Derived Neurotrophic Factor	BDNF
Caesium Chloride	CsCl
Central Nervous System	CNS
Ciliary Neurotrophic Factor	CNTF
complementary DNA	cDNA
c-Src regulatory kinase	Csk
cytosolic PhosphoLipase A_2	$cPLA_2$
deoxyNucleotideTriphosPhosphate	dNTP
DiEthylPyroCarbonate	DEPC
DiMethylFluoride	DMF
DiThioThreitol	DTT
double distilled H_2O	ddH_2O
Embryonic Stem	ES
Epidermal Growth Factor	EGF
Ethanol	EtOH
Ethidium Bromide	EtBr
EthyleneDiamineTetraAcetic acid	EDTA
European Molecular Biological Laboratories	EMBL
Extension Temperature	T_E
Extension Time	ET
Extracellular Regulated Kinases	ERKs
Fibroblast Growth Factor	FGF
Focal Adhesion Kinase	FAK
Glial-cell Derived Neurotrophic Factor	GDNF
G-Protein Coupled Receptor Ligand's	GPCRL's
Growth factor-Receptor binding protein 2	Grb2
GTPase-Activating Protein	GAP
Guanine Di-Phosphate	GDP
Guanine Nucleotide (Exchange/Release) Protein	GN(E/R)P
Guanine Tri-Phosphate	GTP
Hybridisation Temperature	T_H

HydroChloric Acid	HCl
Insulin-like Growth Factor	IGF
Interferon	IFN
Interleukin-3	IL-3
Janus Kinase	JAK
Leukaemia Inhibitory Factor	LIF
Low Affinity Neurotrophin Receptor	LNF
LysoPhophatidic Acid	LPA
Magnesium Chloride	$MgCl_2$
mammalian Son of sevenless	mSos
Melting Temperature	T_M
Mitogen-Activated Protein	MAP
Mutliple Cloning Site	MCS
Myristoylated Alanine-rich C-Kinase Substrate	MARCKS
3-(N-Morpholino)PropanesSulfonic acid	MOPS
Neomycin resistant	Neo^r
Nerve Growth Factor	NGF
Neurotrophin-3	NT-3
Neurotrophin-4	NT-4
New England Biolabs	NEB
Peripheral Nervous System	PNS
PhosphoLipaseC(γ)	PLC(γ)
Plaque Forming Units	PFU
Platlet Derived Growth Factor	PDGF
Pleckstrin Homology	PH
PolyAdenylation	pla
PolyEthylene Glycol	PEG
PolyEthylene Imine	PEI
Positely Charged Nylon Membrane	PCNM
Potassium Acetate	KOAc
Potassium Chloride	KCl
Protein Kinase C	PKC
Retanoic Receptor α1	RARα1
Reverse Transcriptase	RT
Revolutions Per Minute	RPM
Signal Transducers and Activators of Transcription	STAT's
Sodium Acetate	NaOAc
Sodium Chloride	NaCl
Sodium Dodecyl Sulphate	SDS
Sodium Hydroxide	NaOH

Stress activated protein/ERK Kinase-1	SEK-1
Superior Cervical Ganglion	SCG
N,N,N',N'-tetramethylethyldiamine	TEMED
Thymidine Kinase	TK
Transforming Growth Factor	TGF
Tris-Hydrochloride	Tris-HCl
Tris(hydroxymethyl)aminomethane	Tris
Tyrosine Hydroxylase	TH
Tyrosine Kinase 2	Tyk2
UnTranslated Region	UTR
UltraViolet	UV
5-bromo-4-chloro-3-indolyl-β-Dgalactose	X-gal

Table of Contents.

List of Figures

List of Tables

CHAPTER 1 Does $G_{z\alpha}$ act as a Second Messenger in Nerve Cell Signalling?

Part 1.1 Introduction.

The (heterotrimeric) G-protein G_z is predominantly found within neuronal tissues (Hinton *et al.*, 1990) and platelets (Lounsbury *et al.*, 1991), however, it is insensitive to both cholera and pertussis toxins (Matsuoka *et al.*, 1988; 1990), so whatever unique biological role it might play within these tissues has been difficult to ascertain. The α-subunit of G_z ($G_{z\alpha}$) undergoes retrograde transport within nerve fibres (Crouch *et al.*, 1994) and has a slow rate of guanine triphosphate (GTP) hydrolysis (Casey *et al.*, 1990). It has, therefore, been proposed that $G_{z\alpha}$ may act as a stable second messenger, mediating the signal generated by factors at the nerve terminal which aren't themselves transported back to the nucleus (Crouch *et al.*, 1994; Hendry *et al.*, 1995). Furthermore, there is enough evidence to suggest that G-proteins in general play a far wider role than simple effector activation at or near the cell's surface (Malarkey *et al.*, 1995). This means that any biological function assigned to $G_{z\alpha}$, through its disruption *in vivo* (Chapter 3, p55) must be taken in the context of the ever widening role that G-proteins play in cell signalling.

Part 1.2 G-proteins and Cytosolic Signalling.

Section 1.2.1 Introduction

Specific associations between cytosolic signalling pathways and ligand specific membrane spanning receptors have evolved in nature to process and initiate a logical response to extracellular signals. Two widely studied groups include the tyrosine kinase growth factor receptors (Fantl *et al.*, 1993), known to activate/phosphorylate members of the mitogen-activated protein (MAP) kinase cascade (Malarkey *et al.*, 1995), and the G-protein coupled seven transmembrane receptors (Birnbaumer, 1990). These relationships are not, however, exclusive and the degree to which these pathways interact shows that a clear distinction can no longer be drawn between a response such as cell division and the activation of any one group of effectors, or second messengers. Signals generated by both tyrosine kinase and G-protein coupled receptors form complex relationships in order to initiate different biological responses, including growth and development (Otte *et al.*, 1992), neurotransmission (Goh & Pennefather, 1989; Wu *et al.*, 1992), neuronal survival and regeneration (Thoenen, 1991; Curtis *et al*, 1994; Curtis & DiStefano, 1994; Snider, 1994), sensory transduction (Hepler & Gilman, 1992); blood clotting (Manning & Brass, 1991), cardiac function (Luetje *et al.*, 1988; Birnbaumer, 1990), and hormonal regulation (Linder & Gilman, 1992).

Section 1.2.2 The Role of G-proteins in Signal Transduction.

a) GTP Binding & Hydrolysing Proteins (GTPases). The GTPases are intimately involved in many receptor activated pathways (Bourne, 1988). They form a class or family which include: (i) the α subunit of the heterotrimeric G-proteins (G_α; Bourne *et al.*, 1990; 1991); (ii) various translation factors linked to protein synthesis (e.g. the bacterial elongation factor (EF)-Tu; Jurnak, 1985; LaCour *et al.*, 1985); (iii) the 21kDa products of the *ras* family of proto-oncogenes ($p21^{ras}$; DeVos *et al.*, 1988; Milburn *et al.*, 1990; Pai *et al.*, 1989; 1990); (vi) tubulin; and (v) the "small G's", 20-35kDa proteins which function by regulating cell growth, protein secretion and vesicular transport (e.g. products of the *rab*, *rap*, *rho*, *rac*, *smg21*, *smg25*, *YPT*, *SEC4* & *ARF* genes; Bourne, 1988; Balch,

1989; Bourne *et al.*, 1990; 1991; Hall, 1990; Kaziro *et al.*, 1991; von Mollard *et al.*, 1991; Barlowe *et al.*, 1993; Zhang *et al.*, 1993).

b) GTPase Activation/Deactivation: a Common Cycle. When activation occurs the GTP-binding protein, in association with a guanine nucleotide release/exchange protein (GN(R/E)P), replaces bound guanine diphosphate (GDP) for GTP. In this GTP-bound conformation the protein is capable of regulating cell function through the activation of cytosolic effectors. Deactivation occurs when GTP is hydrolysed to GDP. This can occur independently or through the intervention of a class of GTPase-activating proteins (GAP's; Bourne *et al.*, 1990; Kaziro *et al.*, 1991).

c) The G-proteins. G-proteins form a subclass of GTPases which classically mediate the signals generated by a group of seven transmembrane G-protein coupled receptors (GPCR's) and their specific ligands (GPCRL's; Table 1.1; Birnbaumer, 1990). The G-protein complex is a heterotrimer made up of an α, β and γ subunit (Lamb & Pugh, 1992; Linder & Gilman, 1992). Upon receptor activation the G_α subunit (the GTPase, see above) exchanges bound GDP for GTP, leading to the dissociation of the GTP-bound G_α the $G_{\beta\gamma}$ heterodimer. In this activated state the G_α-GTP acts as a cytosolic second messenger and can initiate certain pathways. Several examples include: (i) activation of the retinal cyclic guanine monophosphate (cGMP) specific, phosphodiesterase by the G_α subunit of transducin, $G_{\alpha t}$ (Wheeler & Bitensky, 1977); (ii) stimulation/inhibition of adenylyl cyclase by $G_{s\alpha}/G_{i\alpha}$ (Rodbell *et al.*, 1971; Birnbaumer, 1973; Birnbaumer *et al.*, 1974; Yamamura *et al.*, 1977; Londos *et al.*, 1978); and (iii) ion channel activation by $G_{i\alpha}/G_{o\alpha}/G_{s\alpha}$ (Breitwieser & Szabo, 1985; Pfaffinger *et al.*, 1985). GTP hydrolysis/inactivation of the α-subunit leads to the re-formation of the $G_{\alpha\beta\gamma}$ heterotrimer and a return to complete what has been termed the "G-protein Cycle" (Figure 1.1). Post translational acylation of some G_α's may assist in localising this subunit to the membrane and receptor (Mumby *et al.*, 1990; Casey, 1992), although GDP-bound G_α's are not recognised by their receptor *in vitro* unless accompanied by associated $G_{\beta\gamma}$'s (Hepler & Gilman, 1992). Despite this it is still not entirely certain what the actual biological role of the $G_{\beta\gamma}$ heterodimer is. It is believed that whilst the G_β subunit facilitates receptor coupling (Kleuss *et al.*, 1992), the G_γ subunit, which undergoes post translational

lipidation (Hepler & Gilman, 1992), acts by anchoring the $G_{\beta\gamma}$ complex to the membrane (Yamane *et al.*, 1990; Simon *et al.*, 1991). It has also been suggested that the $G_{\beta\gamma}$ heterodimer individually, or in association, with the G_{α} subunit may play a more general role in mediating/modifying the signal generated by various membrane spanning receptors and other cytosolic effectors (Hepler & Gilman, 1992; Schmidt *et al.*, 1992; Simon *et al.*, 1991; Sternweis & Smrcka, 1992; Tang & Gilman, 1992; Malarkey *et al.*, 1995).

Table 1.1 Ligands Acting Through G-protein Coupled Receptors.

(Reference: Birnbaumer, 1990)

Neurotransmitters & autocoids	Catecholamines; Acetylcholine (M type); Dopamine; Serotonin; Histamine; GABA; Glutamic acid; and Purines.
Peptide & glycoprotein	ACTH; Opioids (>5ligands); MSH, CRF, GRF, TRH, GnRH & SRIF; hormones Vasopressin/oxytocin; Glucagon Glucagon 19-29; CCK, PTH, AngII, GRP, calcitonin, CGRP, NPY, PYY, secretin galanin, kyotorphin; VIP; PHI (PMI); Bradykinin; LH, FSH & TSH; Neurokinins [substance P (NK1), substance K]
Arachidonic acid metabolites	PGE1/2; PGD, PGI_2, thromboxane, LtA_4, LtB4, LtC4, $PGF_{2\alpha}$.
Sensory	light, odor; and taste.
Other	Chemoattractant (Met-Leu-Phe or fMLP), C5a & C3a; thrombine; Phosphatidic acid; and PAF.

d) G-protein Tissue Distribution. A number of G_{α} subunit second messengers have been found to be either tissue or cell type specific, and this may reflect the different functions which they perform. These include: (i) the two forms of transducin, $G_{t\alpha}$ ($T_{c\text{-}\alpha}$) cone and $G_{t\alpha}$ ($T_{r\text{-}\alpha}$) rod in the retina (Leara *et al.*, 1986; Rodrigues *et al.*, 1987; Hepler & Gilman, 1992); (ii) the homolog of $G_{s\alpha}$ (G_{olf}) within the olfactory neurones (Jones & Reed,

1989); and (iii) $G_{o\alpha}$ which is found with the brain and the neuroendocrine system (Perry, 1982; Asano *et al.*, 1988; Chang *et al.*, 1988; Gabrion *et al.*, 1989; Peraldi *et al.*, 1989). Each of these observations has lead to the speculation that $G_{z\alpha}$, which appears to be abundant within neuronal tissues (Hinton *et al.*, 1990), has a specific role to play within nerve cells (Hendry *et al.*, 1996).

Section 1.2.3 Do G-proteins Play a Role in Mediating the Signal Generated by Tyrosine Kinase Growth Factor Receptors?

a) Growth Factor Tyrosine Kinase Receptors. As the name suggests the tyrosine kinase receptors are a class of predominantly growth factor associated receptors, which possess an intrinsic tyrosine kinase catalytic function. Several examples include the platelet derived growth factor (PDGF) receptor, the epidermal growth factor (EGF) receptor and the fibroblast growth factor (FGF) receptor (Fantl *et al.*, 1993). For these receptors the key event following ligand binding is the dimerisation of activated monomeric forms of the receptor and the phosphorylation of specific intracellular tyrosines (Fantl *et al.*, 1993; Kazlauskas & Cooper, 1989). The signal is then transferred to various secondary effectors (e.g. PLCγ, c-Src, Ras, GAP etc, see Table 1.2), through a region of 'Src' homology, designated the Src homology-2 (SH2) domain (Figure 1.1; Matsuda *et al.*, 1990; Pawson & Gish, 1992). Those effectors that contain SH2 domains but which do not themselves appear to possess an intrinsic function (e.g. GRB2, Nck, etc, see Table 1.2) also contain a region of Src homology, the SH3 domain which binds proline-rich sequences (Ren *et al.*, 1993). These 'adaptors' are thought to assist in the activation of those proteins which do not have an intrinsic SH2 domain, through adaptor/SH3 mediated binding to the activated tyrosine kinase receptor (e.g. mammalian Son of sevenless, mSos; Duchesne *et al.*, 1993).

b) Tyrosine Kinase Receptor Activation and the Ras/MAP Kinase Signalling Pathway. The GRB2 'adaptor' (Egan *et al.*, 1993) provides a key link between tyrosine kinase receptors and the Ras/MAP kinase mitogenic signalling pathway, through the activation of the $p21^{ras}$ (see above; Trahey & McCormick, 1987; Hall, 1994) specific GNEP mSos (Bonfini *et al.*, 1992; Bowtell *et al.*, 1992). This is followed by the phosphorylation or

Table 1.2 Proteins Containing SH2 & SH3 Domains.

(Reference: Malarkey *et al.*, 1995)

Protein	SH2	SH3	Function/Targets
PLCγ	Yes	Yes	PtdIns(4,5)P_2 hydrolysis
GAP	Yes	Yes	Ras GTPase
Src	Yes	Yes	Tyr kinase/signalling in haematopoietic cells
SHPTP-1	Yes		Tyrosine phosphatase
SHPTP-2	Yes		Tyr phosphatase/GRB2 adaptor
VAV	Yes	Yes	Nucleotide exchange in haematopoietic cells
ZAP-70	Yes		Tyrosine kinase (T cells)
p72*syk*	Yes		Tyrosine kinase (B cells)
GRB2	Yes	Yes	Adaptor to mSos
p85 PI 3-kinase	Yes	Yes	110kDa PI 3-kinase
Nck	Yes	Yes	Unknown
SHC	Yes		Adaptor to GRB2
Tensin	Yes		Cytoskeletal protein
p47 and p67*phox*		Yes	Cytochrome b 558
α-Spectrin		Yes	Cytoskeletal protein
STAT 91	Yes	Yes	ISGF complex/GAF

activation of a 'cascade' of intermediate cytosolic signalling molecules (Figure 1.1). GTP-bound p21*ras* activates a homolog of the oncogene v-*raf*, the ubiquitously expressed c-*raf*-1 (Dent *et al.*, 1992; de Vries-Smits *et al.*, 1992; Howe *et al.*, 1992; Troppmair *et al.*, 1992; Williams & Robert, 1994) indirectly, by localising it to the membrane to await phosphorylation/activation by some other kinase (Leevers *et al.*, 1994; Stokoe *et al.*, 1994). Activated c-Raf-1 activates/phosphorylates the MAP kinase kinase (MEK; Kyriakis *et al.*, 1992; Yan & Templeton, 1994; Zheng & Guan, 1994a) and MEK phosphorylates/activates the MAP kinases or extracellular regulated kinases (ERK's; Anderson *et al.*, 1990; Gomez & Cohen, 1991; Crews & Erikson, 1992; Nakielny *et al.*, 1992). The Erk's play a number of important biological functions which include, the activation of various transcription factors (e.g. c-*jun*; Pulverer *et al.*, 1991) and cytosolic

effectors (e.g. phospholipase A_2, $cPLA_2$; for review see Bienis, 1993). MAP kinase has also been shown to phosphorylate MEK, a function which may allow it to negatively regulate its own activity (Rossomando *et al.*, 1994). MEK is particularly interesting because it links a number of pathways through protein kinase C (PKC) to the MAP kinase cascade (Figure 1.1). A number of different forms of MEK have been identified (MEK1 & MEK2), as well as the splice variant of MEK1, MEK1b (MEK3; Seger *et al.*, 1992; Zheng & Guan, 1993; 1994a).

c) PKC-dependent GPCRL Activation of the Ras/MAP Kinase Cascade. GPCRL stimulation of polyphospholipid hydrolysis, through PLCβ and the subsequent activation of PKC, has been suggested as one way in which G-protein activation can mediate the signal generated by the Ras/Map kinase cascade (Force *et al.*, 1991; Kahan *et al.*, 1992; McKenzie *et al.*, 1992). Possible sites of action include, MEK (Granot *et al.*, 1993; Bogoyevitch *et al.*, 1994) and/or c-Raf-1 (Kolch *et al.*, 1993), both of which are phosphorylated by PKC. However, it seems reasonable to suggest that PKC may play a more general role, mediating different reponses to a wide variety of cytoplasmic signalling events (Malarkey *et al.*, 1995; Figure 1.1).

d) PKC-independent GPCRL Activation of the Ras/MAP Kinase Cascade. In addition to the activation of MAP kinase through PKC, several GPCRL's appear to be capable of activating the Ras/MAP kinase cascade, through components of the 'G-protein cycle' (Kahan *et al.*, 1992; Cook *et al.*, 1993; van Corven *et al.*, 1993; Hordijk *et al.*, 1994). Both thrombin and the mitogenic phospholipid, lysophophatidic acid (LPA) have the potential to activate $p21^{ras}$ *in vivo*, either: (i) directly through the pertussis toxin sensitive G_{α}, $G_{\alpha i}$, (Gupta *et al.*, 1992; Albias *et al.*, 1993; Gardner *et al.*, 1993; Winitz *et al.*, 1993); or (ii) indirectly through the $G_{i\beta\gamma}$ subunit complex (Faure *et al.*, 1994), which is thought to facilitate the membrane localisation of various Ras regulatory proteins, possessing a region of pleckstrin homology (the PH domain; Koch *et al.*, 1993; Bimey, 1994; Crespo *et al.*, 1994). It is important to note, however, that many of these studies use either constitutively active $G_{i\alpha}$, or over expressed $G_{i\beta\gamma}$, to obtain $p21^{ras}$/MAP kinase activation, so the actual role of these proteins may be to enhance an existing signal rather than to initiate one, by assisting in the activation of $p21^{ras}$ (Malarkey *et al.*, 1995).

e) GPCRL Activation of MAP kinase through the MEK Kinase? The MEK kinase, a 74kDa protein originally identified through its homology to two known MEK kinases in yeasts (STE11 & Byr2; Lange-Carter *et al.*, 1993; 1994a;1994b; Lange-Carter & Johnson, 1994; Zheng *et al.*, 1994; Zheng & Guan, 1994b; Figure 1.1), has been proposed as an alternative downstream regulator of the Ras cascade, which is not dependent upon either PKC or Ras/Raf (Gupta *et al.*, 1992; Burgering *et al.*, 1993; Kizaka-Kondoh & Okayama, 1993). The evidence linking GPCRL's to MEK kinase is largely circumstantial and is based upon the assumption that PKC activation of MEK is not the only avenue for Raf/Ras independent activation of the Ras/MAP cascade (Malarkey *et al.*, 1995). Notably, the over-expression of MEK kinase also results in the specific activation of the stress activated protein/ERK kinase-1 (SEK-1), which is part of another pathway, the 'stress activated' protein (or SAP) cascade (Yan *et al.*, 1994).

Section 1.2.4 Other Tyrosine Kinase Associated Signalling Pathways.

a) The Focal Adhesion Kinase (FAK). FAK is a cytosolic tyrosine kinase, that has been localised to cellular focal adhesion sites, and which, although it does not possess either an SH2 or SH3 domain (Schaller *et al.*, 1992), does interact with a number of secondary effectors including GRB2, PLCγ and p85 (Clark & Brugge, 1993; Schlaepfer *et al.*, 1994; Malarkey *et al.*, 1995). More specifically FAK may act by translocating c-Src to specific membrane locations, for its inactivation by the c-Src regulatory kinase (Csk; Moszcynska & Opas, 1994; Sabe *et al.*, 1994). Two suggested biological roles for FAK include the phosphorylation of various cytoskeletal proteins (Aderem, 1992; Kornberg *et al.*, 1992) and the removal of adherence to the extracellular matrix prior to mitogenesis (Kanner *et al.*, 1990; Guan & Shalloway, 1992). Whatever it's true biological function FAK's close association with integrin GpIIb-IIIa (Lipfert *et al.*, 1992; Fox *et al.*, 1993; Haimovich *et al.*, 1993) makes it a possible link between cytoplasmic signalling pathways and the extracellular matrix (Hanks *et al.*, 1992). It is also interesting to note that the PKC independent phosphorylation of FAK by the GPCRL's, bombesin and endothelin-1 (Zachary *et al.*, 1992; Sinnett-Smith *et al.*, 1993; Saville *et al.*, 1994) has also been linked to the mobilisation of G_γ subunits to focal adhesion sites within the cell (Hansen *et al.*, 1994; Schorb *et al.*, 1994), although the role of G-proteins in FAK activity is unknown.

b) The Tyrosine Kinase2/Janus Kinases. The Tyk2/JAK kinases form a family of cytoplasmic tyrosine kinases (Argetsinger *et al.*, 1993; Darnell *et al.*, 1994) which, like FAK, do not possess either an SH2 or SH3 domain (Firmbach-Kraft *et al.*, 1990; Wilks *et al.*, 1991; Velazquez *et al.*, 1992). Instead they require a set of signal transducers and activators of transcription (STAT's: p84, p91 & p113; Fu *et al.*, 1992) which do, to bind and mediate the signal generated by a class of non-tyrosine kinase receptors. These include the α and γ interferon (α & γ IFN; Schindler *et al.*, 1992; Shuai *et al.*, 1992), interleukin-3 (IL-3; Silvennoinen *et al.*, 1993a), erythropoietin (Witthuhn *et al.*, 1993) and certain growth factor receptors (Argetsinger *et al.*, 1993). The role of JAK/STAT's as activators of transcription, however, may be more widespread as the p91 STAT has also been implicated in the EGF and PDGF stimulated, Ras independent, formation of DNA binding complexes (Fu & Zhang, 1993), two growth factors normally associated with receptor tyrosine kinase activation of the Ras/MAP kinase cascade (Shuai *et al.*, 1992; Ruff-Jamison *et al.*, 1993; Silvennoinen *et al.*, 1993b).

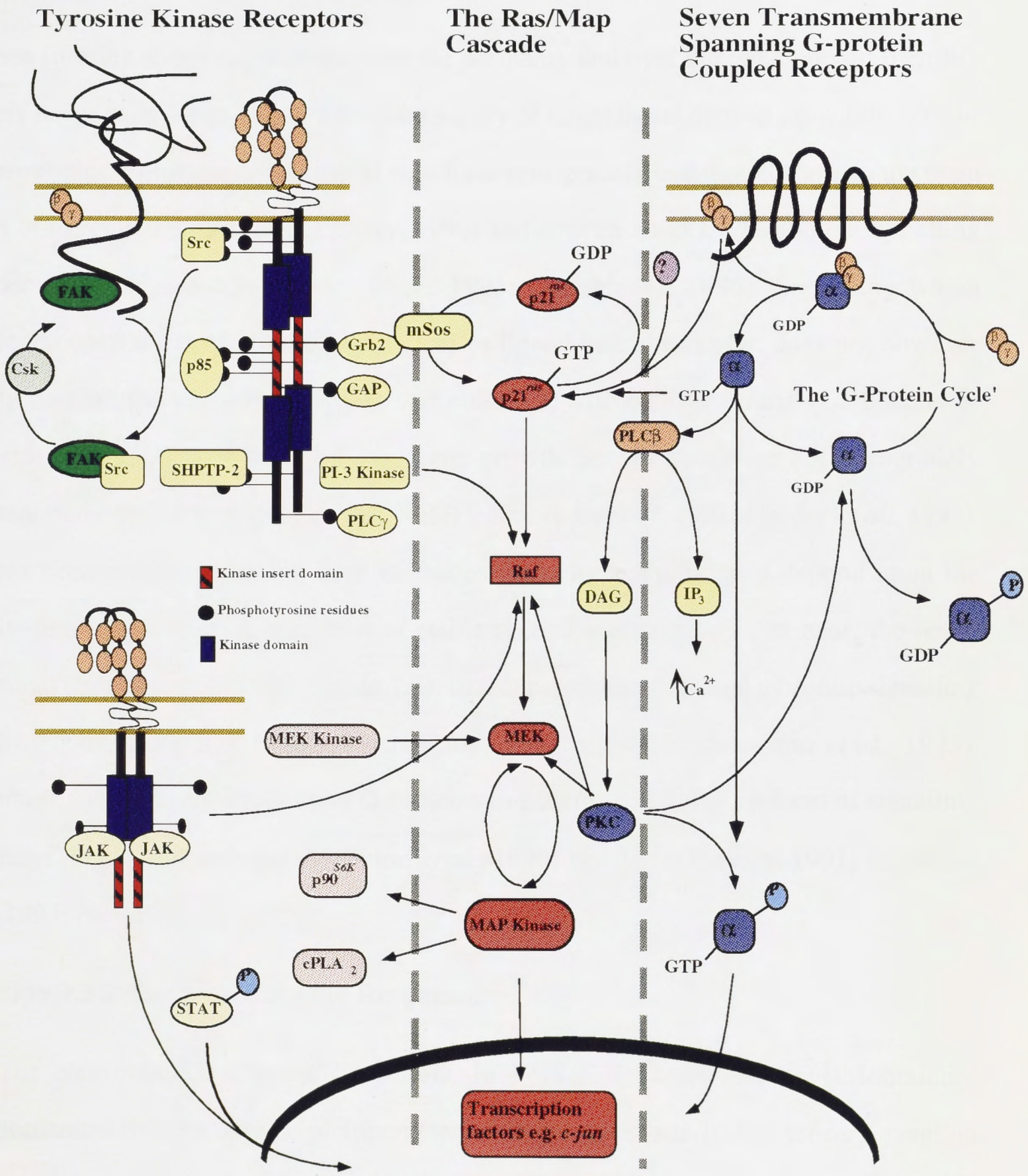
1.2.5 A MAP kinase Independent G-protein Coupled Mitogenic Pathway?

Despite the fact that many of the putative mitogenic pathways detailed above, act principally through cytosolic tyrosine kinases (Yarden & Ulrich, 1988), there may be a G-protein associated mitogenic pathway which is not dependent upon an association with, or the activation of a tyrosine kinase cascade (Crouch & Simpson, 1996). This is controversial because it conflicts with the perceived action of GPCRL's which, if they effect transcription at all, do so through the Ras/MAP kinase pathway via the hydrolysis of phospholipids and the activation of PKC (Brown *et al.*, 1984; Vincentini & Villereal, 1984; Paris & Pouyssegur, 1986; Zachary *et al.*, 1986), and not directly through the components of the G-protein cycle, previously thought to fulfil well defined roles close to the membrane (Birnbaumer, 1990). The idea is worth further examination, however, because it brings together many of the observations which have pointed to a wider role for G-proteins than simple effector activation at or near the cells surface. For instance, $G_{i\alpha}$ has been implicated in the stimulation of mitogenesis by EGF, insulin and post receptor activated PKC by phorbol ester. This places $G_{i\alpha}$ downstream of PKC and is supported by the finding which shows phosphorylation/activation of $G_{i\alpha}$ and the movement of $G_{i\alpha}$

subunits from the membrane to perinuclear sites within the cell prior to EGF stimulated cell division (Crouch *et al.*, 1990; 1991). It has been recently proposed that the nuclear translocation of $G_{i\alpha}$ may be to initiate mitosis, however like many second messengers the full extent of its role in mediating biological function is unknown (Crouch & Simpson, 1996).

Figure 1.1 Tyrosine Kinase Signalling Pathways and G-proteins.

Characteristic of the tyrosine kinase associated family of receptors (Fantl e*t al.*, 1993) is the presence of an extracellular binding domain, a membrane spanning portion and a region of intracellular tyrosine kinase activity, which binds various second messengers including: phospholipaseCγ (PLCγ; Meisenhelder *et al.*, 1989; Ronnstrand *et al.*, 1992); the GTPase-activating protein (GAP; Kazlauskas *et al.*, 1990); the growth factor-receptor binding protein 2 (GRB2; Lowenstein *et al.*, 1992; Bar-Sagi *et al.*, 1993; Arvidsson *et al.*, 1994); the 85kDa subunit of the PI-3 kinase (phosphatidylinositol 3' kinase, p85; Kazlauskas & Cooper, 1989); the phosphotyrosine phosphatase (SHPTP-2; Lachleider *et al.*, 1993); and members of the non-receptor Src family of tyrosine kinases (Mori *et al.*, 1993; Kypta *et al.*, 1990). The 'growth factor associated' tyrosine kinase receptors, possess an intrinsic tyrosine kinase catalytic activity, and can bind these effectors directly. However, other tyrosine kinase-associated membrane spanning receptors do not and require the binding and activation of certain cytosolic tyrosine kinases. These include the focal adhesion kinase (FAK, Schaller *et al.*, 1992) and the Janus kinases (JAKs; Firmbach-kraft *et al.*, 1990; Wilks *et al.*, 1992; Velazquez *et al.*, 1992). Also shown in the figure is the 'G-protein cycle' (Lamb & Pugh, 1992; Linder & Gilman, 1992), activation of PKC and the Ras/MAP kinase cascade. Arrows indication possible points of interaction between each of these cytosolic signalling pathways (Malarkey *et al.*, 1995).


Tyrosine Kinase Receptors
The Ras/Map Cascade
Seven Transmembrane Spanning G-protein Coupled Receptors
Src
FAK
Csk
FAK
Src
p85
Grb2
GAP
SHPTP-2
PI-3 Kinase
PLCγ
mSos
GDP
GTP
Raf
MEK Kinase
MEK
MAP Kinase
p90 S6K
cPLA 2
STAT
P
JAK
JAK
Kinase insert domain
Phosphotyrosine residues
Kinase domain
DAG
IP3
Ca2+
PKC
PLCβ
GDP
GTP
The 'G-Protein Cycle'
GDP
GTP
Transcription factors e.g. c-jun

Part 1.3 Nerve Cell Signalling & Role of Second Messengers.

Section 3.1 Introduction.

When forming stable connections with the periphery and over the remainder of their life many nerve cells depend upon a constant supply of target tissue derived growth factors, or neurotrophic factors (neurotrophins) which are retrogradely transported back to the main part of the cell, the cell body (Hendry, 1992) and activate short lived or labile signalling messengers (Figure 1.2; Hendry *et al.*, 1995; Oppenheim, 1996). Signalling which depends upon the need to transport ligand or ligand bound receptor, does not however fully account for the survival affect that either the extracellular matrix (Carbonetto & David, 1993; David *et al.*, 1995), or some growth factors which are not retrogradely transported have on neuronal cell survival (Hendry & Belford, 1991; Hendry *et al.*, 1992). It has been proposed that in these instances the cells response may depend upon the activation and retrograde transport of stable second messengers at, or near, the nerve terminal (Hendry *et al.*, 1995; Figure 1.2). In fact members of several cytosolic signalling pathways are known to undergo retrograde axonal transport (Johanson *et al.*, 1995), although the evidence implicating G-proteins in general or $G_{z\alpha}$ in this form of signalling remains largely circumstantial (Hinton *et al.*, 1990; Hendry & Crouch, 1991; Crouch *et al.*, 1994; Hendry *et al.*, 1995).

Section 1.3.2 The Neurotrophic Response.

a) The Neurotrophic Theory/Hypothesis. In 1949 Hamburger and Levi-Montalcini, demonstrated that not only do peripheral targets regulate the survival of sensory ganglion neurones in chickens, but that there is also a natural and naturally occurring death of a proportion of these cells. They concluded therefore that during development the ganglion produces more cells and sends out more axons than their peripheral targets could support, and that there was an exchange of metabolic information between the target and the nerve cell (Levi-Montalcini, 1987; Oppenheim, 1996). Implicit in this statement although not fully understood at the time was the basis for the neurotrophic theory, which is that developing neurones compete for limited amounts of target tissue derived 'trophic' factors

that are taken up in the periphery by nerve terminals and conveyed to the cell body where they regulate survival. Subsequent studies in limb removal and tissue transplantation would lead to the eventual discovery of the nerve growth factor (NGF; Hamburger, 1993; Oppenheim, 1996), as well as many other 'neurotrophic' factors (Table 1.3; for review see Curtis & DiStefano, 1994).

Table 1.3 Nerve Growth/Neurotrophic Factors.

Trophic Agent	Abbreviation	References
Nerve Growth Factor	NGF	(see below)
Glial Cell-Derived Neurotrophic Factor	GDNF	Yan *et al.*, 1995
Fibroblast Growth Factor	FGF	Ferguson & Johnson, 1991
Insulin-like Growth Factor	IGF	Ferguson *et al.*, 1991
Brain-Derived Neurotrophic Factor	BDNF	DiStefano *et al.*, 1992 Koliastsos *et al.*, 1993
Neurotrophin-3	NT-3	" "
Neurotrophin-4/5	NT-4/5	" "
Ciliary Neurotrophic Factor	CNTF	Curtis *et al.*, 1993
Leukemia Inhibitory Factor	LIF	Curtis *et al.*, 1994

b) NGF: a Tumour-derived Agent with Unique Physiological Properties. NGF was first isolated from certain tumours known to encourage both sympathetic and sensory nerve fibre innervation, following their transplantation into the coelomic cavity of chick embryos (Bueker, 1948). This response was similar in many ways to that seen following earlier limb transplantation studies, however, there were some marked differences and as the salivary gland appeared to be the sole endogenous source within mice, the delivery of NGF to nerve centres (ganglia) was thought to occur either systemically or through diffusion (Levi-Montalcini & Hamburger, 1953; Hamburger, 1993; Oppenheim, 1996). For these reasons acceptance of the retrograde axonal transport of target tissue derived NGF although central to understanding the biological role of neurotrophic factors as a whole, would have to wait another twenty years and the development of sensitive detection methods not previously available (Oppenheim, 1996).

c) The Retrograde Axonal Transport of Target Tissue Derived NGF. The demonstration that subcutaneously injected radiolabelled NGF accumulated within the sympathetic ganglia of new born mice and that this observation was nerve terminal dependent, would be the first clue to the retrograde axonal transport of this neurotrophic agent (Angeletti *et al.*, 1972). A finding supported by the later observations which showed that (i) NGF is supplied by peripheral targets, which removed the necessity for any single endogenous source (Hendry & Iversen, 1973) and (ii) that NGF directly injected into the anterior chamber of the eye of adult mice accumulates selectively within the cell bodies of its innervating superior cervical ganglion neurones, demonstrating that NGF undergoes retrograde axonal transported (Hendry *et al.*, 1974).

d) NGF: The First Neurotrophin. At the time that the retrograde axonal transport of NGF was considered, the link between NGF and its putative role as a neurotrophic factor, would also be made. In 1973 Hendry and Iversen would suggest that the increased number of neurones observed following NGF administration to young animals was due to the artificial rescue of many of the cells which would normally fail to make contact and die (Hamburger & Levi-Montalcini, 1949; Hendry & Iversen, 1973). This not only helped to link NGF with the trophic signal required for nerve cell survival during development, but also provide a possible source for the 'metabolic' communication between peripheral target and innervating neurone, suggested by Hamburger and Levi-Montalcini 24 years earlier. Subsequent studies have since shown that the role and retrograde transport of NGF within neurones is widespread (Ebbott & Hendry, 1978; Max *et al.*, 1978; Brunso-Bechtold & Hamburger, 1979; Clark, 1982; Seiler & Thoen, 1984; Yan *et al.*, 1988; Wayne & Heaton, 1990) and have confirmed that the peripheral target is the origin of NGF protein and messenger RNA (mRNA; Heumann *et al.*, 1984; Shelton & Reichardt, 1984; Korsching & Thoenen, 1988; Carmignoto *et al.*, 1991).

Section 1.3.3 The Neurotrophic Factors.

a) Introduction. There are two broad groups of neurotrophic factors (Curtis & DiStefano, 1994). The neurotrophins (e.g. NGF, BDNF, NT-3 & NT-4/5; see Table 1.3), are widely expressed in peripheral tissues and mediate nerve cell survival and nerve fibre innervation

during development, and the cytokine-like neurotrophic factors (or neuroactive cytokines, e.g. LIF & CNTF; Table 1.3; Thoenen, 1991; Curtis *et al.*, 1994), which are released by non-neuronal cells in response to neural trauma and may therefore act to promote nerve regeneration and survival following injury. In either case specific receptors mediate the retrograde transport and internalisation of the neurotrophic signal at the nerve terminal (Curtis & DiStefano, 1994; Hendry *et al.*, 1995).

Table 1.4 The Neurotrophin/Trk Receptors.

(for reviews see Meakin & Shooter, 1992; Bothwell, 1995)

Receptor	High Affinity Ligand	Other Interactions
TrkA	NGF	NT-3, NT4/5 (Barbacid, 1994; Berkemeier *et al.*, 1991)
TrkB	BDNF & NT-4/5	NT-3 (Barbacid, 1994)
TrkC	NT-3	-

b) The Neurotrophins. Neurotrophins bind a group of high affinity receptors (the Trk's; TrkA, TrkB, and TrkC; Table 1.4; Meakin & Shooter, 1992; Curtis & DiStefano, 1994; Bothwell, 1995), which possess intrinsic tyrosine kinase catalytic domains and regulate gene transcription through the phosphorylation of cytosolic transcription factors such as CREB (see above; Ghosh *et al.*, 1994; Ginty *et al.*, 1994). Each neurotrophin is also known to bind the low affinity neurotrophin receptor (LNTR, p75) which, does not possess an internal tyrosine kinase catalytic domain (Meakin & Shooter, 1992; Curtis & DiStefano, 1994; Bothwell, 1995). It has been suggested that the LNTR may play a selective role in the retrograde transport of neurotrophins from certain peripheral tissues, although its function remains unclear (Von Bartheld *et al.*, 1994; Curtis *et al.*, 1995; Bothwell, 1995).

c) The Neurotrophin Knockouts. To investigate further the biological role of neurotrophins, mice possessing null gene or null receptor gene mutations, have been created (for review see Snider, 1994). Unsurprisingly, many of these 'gene knockouts' fail to develop properly and die within a month of birth. Nonetheless those that have survived

suggest a degree of ligand redundancy [e.g. BDNF(-/-) & NT-4/5(-/-); Ernfors *et al.*, 1994; Jones *et al.*, 1994; Cohen-Cory & Fraser, 1995; Liu *et al*, 1995] and the severe developmental abnormalities exhibited by the Trk receptor knockouts (Table 1.5) have reinforced the importance of the receptor in mediating the neurotrophic response.

Table 1.5 The Trk Knockouts.

Knockout	Observed Phenotypic Changes
TrkA (-/-)	Survival of certain NGF-dependent neurones (Dreyfus, 1989; Hefti, 1986). Mice are insensitive to heat & pain (Smeyne *et al.*, 1994)
TrkB (-/-)	Severe motor neurone deficiencies significant loss of trigeminal neurons (Klein *et al.*, 1993)
TrkC (-/-)	Death soon after birth, & abnormal motor behaviour (Klein *et al.*, 1994)

d) The Neuroactive Cytokines. The cytokine-like neurotrophic factors (Thoenen, 1991; Curtis *et al.*, 1994) activate a class of receptors which do not possess intrinsic tyrosine kinase catalytic domain (e.g. Lifβ & gp130; Stahl & Yancopoulos, 1992). In this instance whilst agonist stimulation results in the formation of a heterodimer and activation of the MAP kinase cascade, these class of receptors also stimulate the phosphorylation of JAK/STAT's family of cytoplasmic second messengers (Bonni *et al.*, 1993; Boulton *et al.*, 1994; Darnell *et al.*, 1994).

e) The Non-Transported 'Trophic' Factors. Several factors which are not retrogradely transported within neurones but which can still be said to have a neurotrophic effect on the cell include the extracellular matrix (e.g. Laminin which binds the integrin receptor and may through FAK activate a number of cytosolic secondary signalling molecules; see above; Carbonetto & David, 1993; Fox *et al.*, 1993; Schlaepfer *et al.*, 1994; David *et al.*, 1995) and several neurotrophic factors, whose retrograde transport appears to be either entirely absent or cell-type specific. For example, even though FGF is required for the survival of neurons in culture, the regeneration of injured axons (Dreyer *et al.*, 1989;

Grothe *et al.*, 1989) and development of the parasympathetic nervous system (Hendry *et al.*, 1988), it does not appear to undergo retrograde axonal transport (Hendry & Belford, 1991). Furthermore whilst the neuroactive cytokine LIF promotes the survival of sensory neurons (Murphy *et al.*, 1991) and the differentiation of sympathetic neurons from the adrenergic to the cholinergic phenotype, it only appears to be transported within the sensory neurones (Hendry *et al.*, 1992).

f) Summary. Recently several neurotrophins have been implicated in aspects of nerve cell growth and maintenance which go beyond the simple survival role they might play during development, including nerve regeneration (Hagg *et al.*, 1990; Raivich & Kreutzberg, 1993), maturation (Knusel *et al.*, 1995; Wang *et al.*, 1995) and proliferation (Gaese *et al.*, 1994). What these observations suggest is that the distinctions made between neurotrophins and the cytokine like neurotrophic factors, although convenient, are not based upon large functional differences. Furthermore, because each growth factor receptor has the potential to activate many shared cytoplasmic signalling pathways (see above), it is not unreasonable to suggest that a cell's response to ligand activation is due to the combined effect of a large number of different, often competing signals and not necessarily the arrival of any single messenger. This provides a new avenue for understanding the role of neurotrophins, for whilst receptor knockouts may prove fatal and ligand knockouts redundant, subtle phenotypes generated through the disruption of putative second messengers may prove more useful in the longer term to understanding the function of neurotrophic factors in living systems (for possible 'gene targeting' outcomes see Chapter 5, p109).

Section 1.3.4 Models For Second Messenger Activation.

a) Introduction. Neurones face a special problem in conveying signals generated at the nerve terminal because of the distance between the site of ligand binding (the nerve terminal) and the nucleus. This has lead to the development of models seeking to describe systems of active transport in which the growth factor, or second messenger is retrogradely transported to the cell body for processing (for reviews Curtis & DiStefano, 1994; Hendry *et al.*, 1995; Oppenheim, 1996).

b) The Labile Second Messenger Model. In most instances neurotrophic factors are retrogradely transported within cytosolic vesicles and presumably along microtubules back to the cell body, where they mediate the activation of various cytosolic signalling pathways (Curtis & Distefano, 1994; Snider, 1994). Whilst ligand binding is important for receptor activation it is the activated receptor which directly mediates both vesicle formation and subsequent effector activation through its intracellular tyrosine kinase catalytic domain (Heumann *et al.*, 1981; Johnson *et al.*, 1989; Distefano *et al.*, 1992; Curtis *et al.*, 1994). This has been termed the labile second messenger (LSM; Figure 1.2) model because it accounts for the activation and action of short lived cytosolic effectors close to the nucleus (Hendry *et al.*, 1995). The value of this model is that it may also describe the retrograde transport of cytosolic proteins that use other means of attachment to the vesicle membrane, such as fatty acylation or hydrophobic interactions (Curtis & DiStefano *et al.*, 1994).

c) The Stable Second Messenger Model. There may, however, be another means of signal generation, which does not depend upon the internalisation and transport of the of the ligand. Neurotrophic factors and components of the extracellular matrix that are not themselves retrogradely transported (see above), may still convey a signal to the cell body through the generation of stable messengers. This has been termed the stable second messenger model (SSM; Figure 1.2; Hendry *et al.*, 1995) and although the identity of these second messenger is unknown, some possibilities have recently been canvassed (Hendry & Crouch, 1991; Crouch *et al.*, 1994; Johanson *et al.*, 1995)

d) Putative Stable Second Messengers. Several cytosolic proteins which have been implicated in a number of signalling pathways undergo retrograde axonal transport (Johanson *et al.*, 1995). These include: (i) members of the Ras/MAP kinase signalling cascade, ERK, MEK (Traverse *et al.*, 1992) and MEK kinase (see above); (ii) the Growth Associated Protein-43 (GAP-43) which has been implicated in nerve fibre regeneration (Tetzlaff *et al.*, 1989); and (iii) the PI-3 kinase (Soltoff *et al.*, 1992), thought to be either directly involved in vesicle formation (Joly *et al.*, 1994; Wennstrom *et al.*, 1994), or as a regulator of membrane trafficking (DeCamilli *et al.*, 1996), through its products, the polyphosphatidylinositides (Whitman *et al.*, 1988; Auger *et al.*, 1989). PI-3 kinase also

binds p21^{ras} (Roche *et al.*, 1994), and it's activity may be enhanced through association with free $G_{\beta\gamma}$'s (Stephens *et al.*, 1993; Stephens *et al.*, 1994). It is likely that both PI-3 kinase and GAP-43 play a role in the formation of signalling complexes at the nerve terminal (Figure 1.3), however, just because something is retrogradely transported does not necessarily mean that it is involved in mediating a stable signal.

e) Do G-proteins act as Stable Second Messengers for Neurotrophic Factors that do not undergo Retrograde Axonal Transport? The survival effect which FGF has on ciliary neurons in culture and the development of sensory and sympathetic neurons *in vivo*, is in both cases at least partly inhibited by pertussis toxin (Hendry & Crouch, 1991). Whilst these observations may not mean that neurotrophic factors act through a pertussis toxin sensitive G-protein such as $G_{i\alpha}$ or any other G_{α}, such a conclusion can be supported by the demonstration that both $G_{i\alpha}$ and the nerve cell specific $G_{z\alpha}$ both undergo retrograde axonal transport within nerve fibres (Hendry & Crouch, 1991; Crouch *et al.*, 1994).

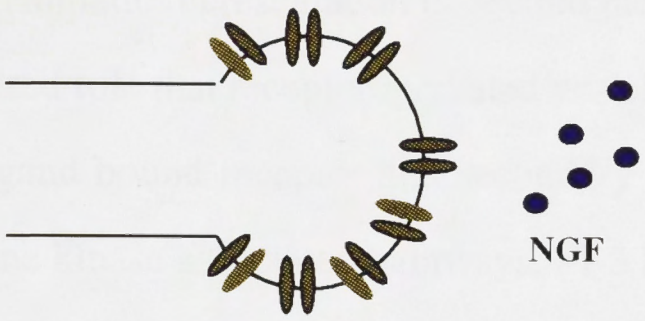
Figure 1.2 Second Messenger Models.

Two models for second messenger activation within nerve cells.

(A) The labile second messenger (LSM) model, which describes the activation of labile or short lived second messengers close to the nucleus.

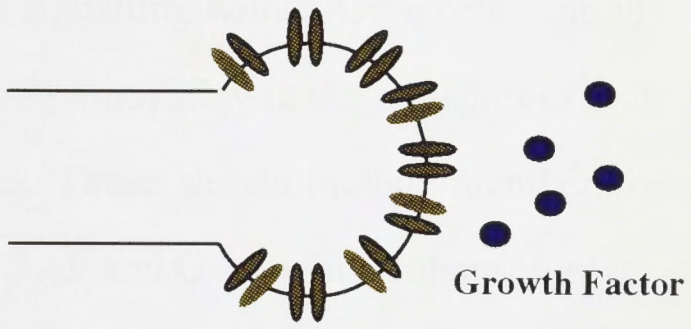
(B) The stable second messenger (SSM) model, which may be utilised by those neurotrophic factors which do not undergo retrograde axonal transport themselves (Figure taken from Hendry *et al.*, 1995).

A

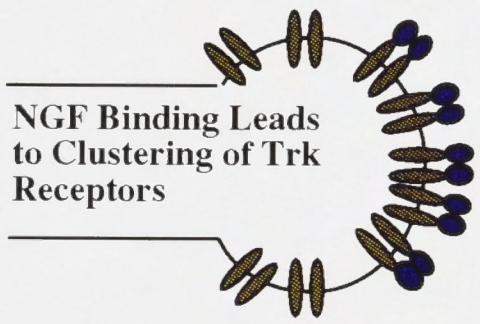


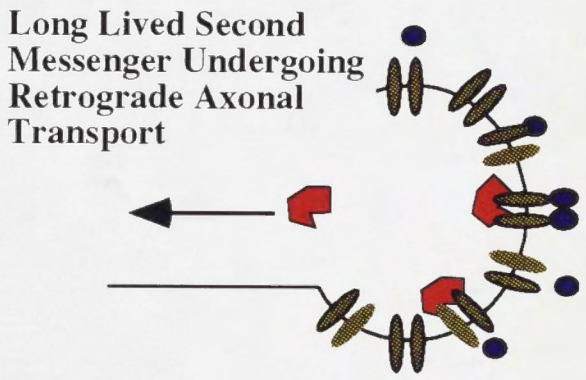
Nerve Terminal

B

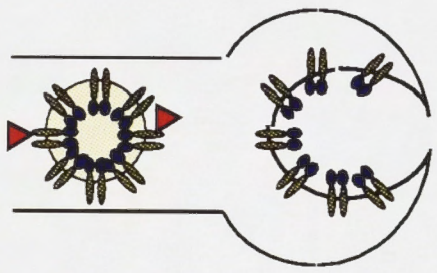


Nerve Terminal

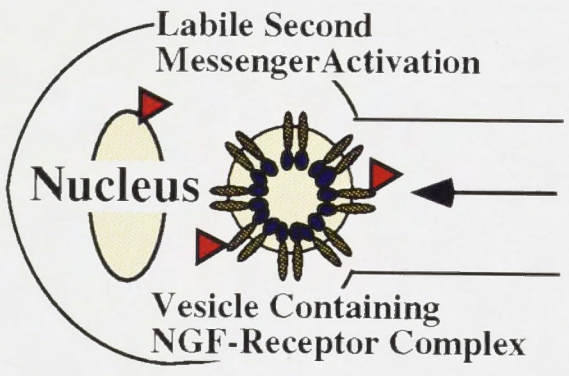




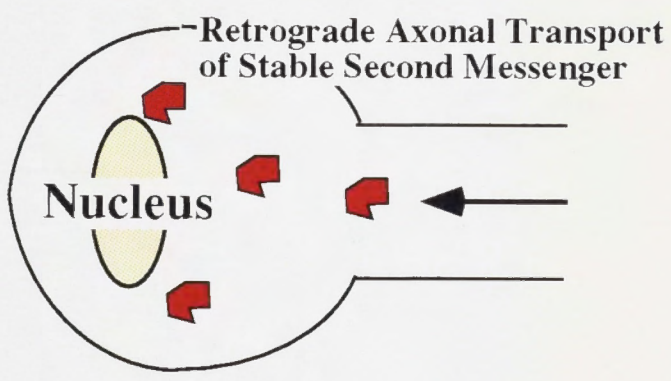
Growth Factor Receptor Complex
May activate both Labile and Stable
Second Messengers



Vesicle Formation and
Retrograde Axonal
Transport of NGF



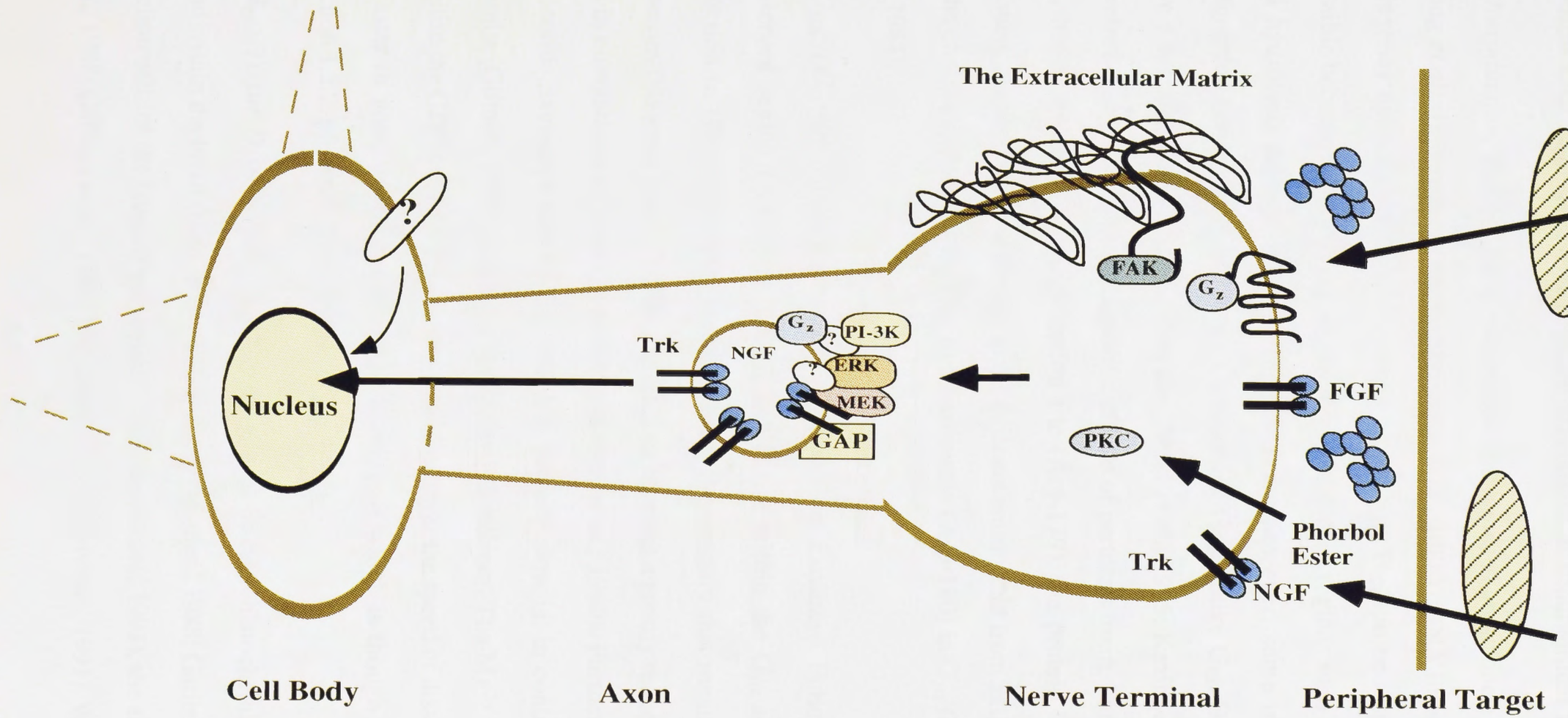
Cell Body



Cell Body

Figure 1.3 Second Messenger Signalling Within Nerve Cells.

Diagrammatic representation of second messenger signalling within nerve cells, and the proposed role that receptor mediated vesicle formation may play in the transport of both the ligand bound receptor and secondary effectors. Those shown include members of tyrosine kinase associated pathways, PI-3 kinase, GAP and G-proteins (Johanson *et al.*, 1995; Hendry *et al.*, 1995).


The Extracellular Matrix
?
Nucleus
Trk
NGF
G_z
?
PI-3K
?
ERK
MEK
GAP
FAK
G_z
FGF
PKC
Phorbol
Ester
Trk
NGF
Cell Body
Axon
Nerve Terminal
Peripheral Target

Part 1.4 The Neurone Specific G-protein: $G_{z\alpha}$.

Section 1.4.1 $G_{z\alpha}$: a G_{α} with Unique Structural & Biochemical Properties.

a) Introduction. The $G_{z\alpha}$ gene was originally identified and cloned from human retinal (Fong *et al.*, 1988) and rat brain (Matsuoka *et al.*, 1988) cDNA libraries, following low stringency hybridisation with bovine $G_{t1\alpha}$ and a rat $G_{i2\alpha}$ probe respectively. This was possible because the highly conserved nature of certain regions such as the GTP-binding and hydrolysis domain (Figure 1.4). There are however, some important structural differences between $G_{z\alpha}$ and other G_{α} subunits. $G_{z\alpha}$, unlike $G_{i\alpha}$, $G_{o\alpha}$, or $G_{t\alpha}$ does not have a functional ADP-ribosylation site (West *et al.*, 1985; Kaziro *et al.*, 1991), and is therefore insensitive to the antagonistic effects of pertussis toxin. Furthermore, although $G_{z\alpha}$ has a cholera toxin modification site (Arg-179), the protein itself appears to be resistant, possibly due to a change in the adjacent amino acid from valine which is present in the cholera toxin sensitive $G_{t\alpha}$, to aspartic acid (Asp-180) in $G_{z\alpha}$ (Figure 1.4; Fong *et al.*, 1988).

b) $G_{z\alpha}$: a G_{α} with a Slow Rate of GTP-Hydrolysis & Exchange. Following mutation of a conserved region (A-G-E to T-S-N; Table 1.4) within the $G_{s\alpha}$ subunit a fifty fold reduction in GTP-hydrolysis was observed. Presumably this means that $G_{z\alpha}$, which possesses this same motif (T-S-N; Figure 1.4), also has a greatly reduced GTP hydrolysis rate in comparison to other G_{α} subunits (Casey *et al.*, 1990). Furthermore, the guanine nucleotide exchange rate of $G_{z\alpha}$ which is already slow is, in contrast with other G_{α} subunits (Gilman, 1987), inhibited further by magnesium. The Mg^{2+} ions are thought to stabilise the GDP-bound form of the protein, however the speed of dissociation is likely to be faster *in vivo*, when catalysed by the receptor which is though to act as a GNEP (Section 1.2.2, p3; Ross, 1989; Parker *et al.*, 1991).

c) $G_{z\alpha}$: Tissue Distribution. Through the use of immunohistochemistry $G_{z\alpha}$ has been found within the brain (Fong *et al.*, 1988; Matsuoka *et al.*, 1988; Garibay *et al.*, 1991), the ganglion cells of the retina (Fong *et al.*, 1988; Jiang *et al.*, 1991), the adrenal gland (Fong *et al.*, 1988; Garibay *et al.*, 1991) and platelets (Gagnon *et al.*, 1991). Within the brain it is

Figure 1.4 Comparison of $G_{z\alpha}$ amino acid sequence with other G_{α}'s.

(A) The region implicated in GTP-hydrolysis, showing the amino acid change A-G-E to T-S-N, which is though to influence the slow rate of GTP-hydrolysis exhibited by recombinant $G_{z\alpha}$. **(B)** The cysteine residue four amino acids from the left is the proposed site of ADP-ribosylation. Like $G_{s1\alpha}$ and $G_{s2\alpha}$, $G_{z\alpha}$ lacks this residue. **(C)** The arginine at position 179, present in all the G_{α} sequences shown, is thought to be the site of cholera toxin modification, however, only $G_{t\alpha}$ and $G_{s\alpha}$ are sensitive. Note the aspartic acid at position 180 within the $G_{z\alpha}$ sequence, which is thought to hinder modification by cholera toxin. The figure below is adapted from Kaziro *et al.*, 1991; Matsuoka *et al.*, 1988. The amino acids are represented by the standard one-letter IUPAC codes.

A Region of GTP binding and hydrolysis.

$G_{z\alpha}$	35-50	K	L	L	L	L	G	**T**	**S**	**N**	S	G	K	S	T	I	V
$G_{i1\alpha}$	35-50	K	L	L	L	L	G	A	G	E	S	G	K	S	T	I	V
$G_{i2\alpha}$	35-50	K	L	L	L	L	G	A	G	E	S	G	K	S	T	I	V
$G_{i3\alpha}$	35-50	K	L	L	L	L	G	A	G	E	S	G	K	S	T	I	V
$G_{o1\alpha}$	35-50	K	L	L	L	L	G	A	G	E	S	G	K	S	T	I	V
$G_{t1\alpha}$	31-46	K	L	L	L	L	G	A	G	E	S	G	K	S	T	I	V
$G_{t2\alpha}$	35-50	K	L	L	L	L	G	A	G	E	S	G	K	S	T	I	V
$G_{s1\alpha}$	42-57	R	L	L	L	L	G	A	G	E	S	G	K	S	T	I	V
$G_{s2\alpha}$	44-59	R	L	L	L	L	G	A	G	E	S	G	K	S	T	I	V

B Putative site of ADP-ribosylation.

$G_{z\alpha}$	346-355	N	L	K	Y	**I**	G	L	C
$G_{i1\alpha}$	345-354	N	L	K	D	C	G	L	F
$G_{i2\alpha}$	346-355	N	L	K	D	C	G	L	F
$G_{i3\alpha}$	345-354	N	L	K	E	C	G	L	Y
$G_{o1\alpha}$	345-354	N	L	R	G	C	G	L	Y
$G_{o2\alpha}$	345-354	N	L	R	G	C	G	L	Y
$G_{t1\alpha}$	341-350	N	L	K	D	C	G	L	F
$G_{t2\alpha}$	385-394	N	L	K	D	C	G	L	F
$G_{s1\alpha}$	385-394	H	L	R	Q	Y	E	L	L
$G_{s2\alpha}$	372-381	H	L	K	Q	Y	E	L	L

C Putative Cholera Modification Site.

	178-180	S	R	**D**
	177-179	T	R	V
	178-180	T	R	V
	177-179	T	R	V
	178-180	T	R	V
	173-175	S	R	V
	178-180	S	R	V
	200-202	C	R	V
	187-189	C	R	V

particularly abundant within pituitary cells (Paulssen *et al.*, 1991), neurons of the basal ganglia, purkinje cells of the cerebellum, granule and pyramidal cells of the hippocampus, the large neurons of layers III and V in the neocortex (Hinton *et al.*, 1990), some neurones within the hippocampus and the cerebral cortex. $G_{z\alpha}$ has also been found within the sciatic nerve (Hendry, 1992; Crouch *et al.*, 1994), sympathetic ganglia (Hinton *et al.*, 1990), the adrenal medulla (Casey *et al.*, 1990), the anterior horns of the spinal cord (Hinton *et al.*, 1990) and *in vitro* within several tumourogenic cell lines. These include: the pheochromocytoma cell line PC12 (Matsuoka *et al.*, 1988, Garibay *et al.*, 1991); several neuroblastoma cell lines (SK-N-SH & SH-EP; Garibay *et al.*, 1991); a neuroblastoma-glioma hybrid (NG108-15; Garibay *et al.*, 1991); a hepatoma cell line (Hep G6; Garibay *et al.*, 1991); and various pituitary cell lines (GH3, GH4C1 & GH12C1; Matsuoka *et al.*, 1988; Garibay *et al.*, 1991; Paulssen *et al.*, 1991).

d) Other Regions of $G_{z\alpha}$ Localisation. There are a number of non-neuronal tissues and organs which may also express $G_{z\alpha}$, including the heart, kidney and erythrocytes (Fong *et al.*, 1988; Spicher *et al.*, 1988; Premont *et al.*, 1989; Garibay *et al.*, 1991; Paulssen *et al.*, 1991), however, this has been disputed by a number of groups (Fong *et al.*, 1988; Matsuoka *et al.*, 1988; Casey *et al.*, 1990; Hinton *et al.*, 1990; Garibay *et al.*, 1991) and its apparent localisation to these tissues may be due to the identification of an as yet undefined $G_{z\alpha}$ homolog, or contamination of the tissue sample by innervating neurones and/or blood platelets (see above).

e) The Retrograde Axonal Transport of $G_{z\alpha}$. Not only is $G_{z\alpha}$ retrogradely transported within nerve fibres (Crouch *et al.*, 1994), but there is some evidence to suggest that this transport also occurs in association with, or is mediated by the cytoskeleton and any internalised vesicles. A theory supported by: (i) the localisation of $G_{z\alpha}$ to microtubules and 'granular' structures within both purkinje cells and sciatic nerve fibres (Hinton *et al.*, 1990; Crouch *et al.*, 1994); and (ii) the copurification of $G_{z\alpha}$ protein, from bovine brain, with a small GTPase implicated in vesicle transport (Section 1.2.2, p3; Casey *et al.*, 1990; Trimble *et al.*, 1991; von Mollard *et al.*, 1991; Barlowe *et al.*, 1993). $G_{z\alpha}$ also undergoes myristolation *in vitro* (Mumby *et al.*, 1990), which may allow it to attach to the membrane of internalised vesicles and interact with other membrane bound effectors (Buss *et al.*,

1987). It must be noted, however, that although this appears to be the most likely means by which $G_{z\alpha}$ is retrogradely transported, evidence for the vesicle mediated retrograde axonal transport of $G_{z\alpha}$ is still largely circumstantial.

Section 1.4.2 A Biological Function for $G_{z\alpha}$?

a) Does $G_{z\alpha}$ act as a Stable Second Messenger? According to the SSM, outlined earlier (Section 1.3.4, p18) for $G_{z\alpha}$-GTP to act as a stable second messenger within neurotrophic signalling it must not only undergo active, specific and retrograde axonal transport within nerve fibres (see above), but it must also be stable enough to reach the cell body while activated. $G_{z\alpha}$ does have a very slow intrinsic rate of GTP-hydrolysis (Casey *et al.*, 1990) which suggests that it can activate effectors far from the site of receptor activation, although it is unknown whether or not this means that $G_{z\alpha}$ can mediate a signal the large distance from the nerve terminal to the cell body. Additionally, the GAP (Section 1.2.2, p3) activity of some G-protein effectors (Bourne & Stryer, 1992), when combined with its slow rate of guanine nucleotide exchange, may also confer a degree of sensitivity to whatever signalling system $G_{z\alpha}$ is involved in.

b) The Role of PKC Phosphorylation in $G_{z\alpha}$ Signalling? Another property of $G_{z\alpha}$ is that it is an effective substrate for phosphorylation by PKC (see also Section 1.2.3, p6; Carlson *et al.*, 1989; Lounsbury *et al.*, 1991). This has been demonstrated both *in vitro* and in intact platelets, treated with thrombin and other PKC activating agents. However, it is not known what affect phosphorylation by PKC has on the activity $G_{z\alpha}$. It may be that PKC phosphorylation of $G_{z\alpha}$ may act to generate a novel signal, however, it is more likely that, because the putative PKC phosphorylation site (Ser-27; Lounsbury *et al.*, 1993) lies within a region known to facilitate binding to the $G_{\beta\gamma}$ heterodimer (Conklin & Bourne, 1993), that PKC phosphorylation of GDP-$G_{z\alpha}$ acts to prevent reassembly of the G-protein $G_{z\alpha\beta\gamma}$ heterotrimer, rapidly attenuating any signal generated through this $G_{z\alpha}$ pathway following effector activation. Furthermore, because activation of $G_{z\alpha}$ may directly or indirectly lead to the activation of PKC (Figure 1.1), PKC phosphorylation of $G_{z\alpha}$-GDP may act as a method of regulating its own activity (Caron & Lefkowitz, 1993; Fields & Casey, 1995).

c) PKC: Clues to a Possible Biological Function for $G_{z\alpha}$. In platelets PKC activation leads to a number of responses which include: (i) the attenuation of adrenaline and thrombin induced inhibition of cyclic AMP accumulation (Jakobs *et al.*, 1985; Watanabe *et al.*, 1985; Carlson *et al.*, 1989); (ii) the inhibition of platelet activating factor mediated increases in cytosolic calcium (MacIntyre *et al.*, 1985); and (iii) the loss of thromboxane A2 activated PLC accumulation (Carlson *et al.*, 1989). $G_{z\alpha}$ is not, however, one of those G-proteins which is thought to couple with either the α-2 adrenergic receptor (Simonds *et al.*, 1989) or the thromboxane A2 receptor (Shenker *et al.*, 1991). Other functions for $G_{z\alpha}$ in platelets may be to control platelet formation and/or megakaryocyte maturation (Manning & Brass, 1991). Within the hippocampus PKC has been implicated in long term potentiation (LTP; Bliss & Collingridge, 1993) and a pertussis toxin insensitive G-protein has also been associated with short term potentiation (STP; the first phase of LTP; Goh & Pennefather, 1989). Unfortunately no direct link between $G_{z\alpha}$, PKC, and a definite biological function has been established within either of these systems.

Section 1.4.3 Summary.

Despite many *in vitro* experiments (Casey *et al.*, 1990; Wong *et al.*, 1992; Shum *et al.*, 1995) it is still not clear which receptors activate $G_{z\alpha}$ *in vivo*, because of its insensitivity to both cholera or pertussis toxin. The unique biochemical properties of $G_{z\alpha}$, however, make it an ideal candidate for stable second messenger activation within nerve cells, something which is enhanced by its tissue distribution (Hinton *et al.*, 1990), retrograde axonal transport (Crouch *et al.*, 1994) and its selective accumulation within the nuclei of sensory neurons (Hendry *et al.*, 1995; Hendry *et al.*, 1996). Like $G_{i\alpha}$, $G_{z\alpha}$ also posses a site for phosphorylation by PKC, and it is interesting to speculate upon whether or not this interaction may be the point at which apparently distinct signalling mechanisms, the seven transmembrane and tyrosine kinase receptor associated signalling pathways, converge. However, there is as yet no direct evidence linking $G_{z\alpha}$ with a typical tyrosine kinase signalling pathway associated response such as nerve cell survival or axon regeneration.

Part 1.5 Project Goals.

Section 1.5.1 General Aims.

In the past, several methods have been used to identify the function of G-proteins *in vivo*. Toxins provide the clearest and simplest way to disrupt function, whilst antisense mRNA targeted oligonucleotides have been used to stop translation of functional protein in tissue culture and transgenic animals (e.g. $G_{z\alpha}$; Moxham *et al.*, 1993). Unfortunately, two of the G-protein 'specific' toxins (e.g. pertussis and cholera toxin; Matsuoka *et al.*, 1988; 1990) appear to have no affect on the intrinsic function of $G_{z\alpha}$, whilst the use of antisense oligonucleotides to target $G_{z\alpha}$ mRNA has lead to inconclusive results (Sven Johanson, personal communication). A gene targeting approach was, therefore, chosen to stably and specifically disrupt expression of the $G_{z\alpha}$ gene (see Part 3.1, p56).

Section 1.5.2 Specific Aims.

The specific goals of the project include:

(i) To design a strategy that can be used to target the expression of the $G_{z\alpha}$ gene *in vivo*.

(ii) To clone and completely characterise a portion of the mouse genome encompassing the $G_{z\alpha}$ gene (Part 3.2, p65).

(iii) To create a mouse $G_{z\alpha}$-specific control construct, that can be used as the control for screening homologous recombinants *in vivo* using the polymerase chain reaction (PCR; Section 3.1.3, p58).

(iv) To create a mouse $G_{z\alpha}$-specific targeting construct, that can be used to disrupt expression of the $G_{z\alpha}$ gene in C57BL/6 mouse embryonic stem (ES) cells (Part 3.3, p86).

(v) To complete the characterisation of the mouse $G_{z\alpha}$ gene including its genomic organisation, with the eventual aim of obtaining a full length cDNA copy of the mRNA (Chapter 4, p98).

CHAPTER 2 Materials and Methods.

Part 2.1 Reagents and Media.

Section 2.1.1 Bacterial Strains.

Escherichia coli DH5α [*endA1 hsdR17* ($r_K^-m_K^+$)*supE44 thi-1 recA1 gyrA* (Nal^r)*relAl Δ(lacZYA-argF) U169 deoR* (ϕ80 *dlacΔ(lacZ) M15)* (NEB, Woodcock *et al.*, 1989)]

E. coli JM110 [F' *traD36lacI^{q}Δ(lacZ)M15 proA$^+$B$^+$/rpsL (Str^r) thr leu thi lacY galK galT ara fhuA dam dcm supE44Δ(lac-proAB)* ; NEB, Yanish-Perron *et al.*, 1985]

E. coli XL1-Blue [F'::Tn10 *proA$^+$B$^+$lacI^q Δ(lacZ) M15/recA1 endA1 gyrA96* (Nal^r) *thi hsdR17* ($r_K^-m_K^+$) *supE4 relAl lac*, NEB]

Section 2.1.2 Enzymes.

a) Modification Enzymes. DNA Polymerase I, Large (Klenow) Fragment; *Thermus aquaticus* (TAQ) DNA Polymerase; T_4 DNA Ligase; T7 Sequenase; Calf Intestinal Phosphatase (CIP); and T_4 Polynucleotide Kinase (Sambrook *et al.*, 1989, NEB)

b) Restriction Enzymes. Restriction endonucleases used in each instance are indicated in the text and were purchased from either Promega or NEB.

Section 2.1.3 Cloning Vectors and Genomic Subclones.

pBluescript II (pB) SK/KS(+/-) phagemid (Appendix 2.1), cloning vectors provided by kindly Dr Klaus Matthaei.

pBSK(+)$G_{z\alpha}$P(696bp, 4BF/3R), the partial $MG_{z\alpha}$ cDNA clone constructed by Leck, 1993 and kindly provided by Dr Klaus Matthaei.

pBSK(+)$G_{z\alpha}$P(mimic) a derivative of the partial $MG_{z\alpha}$ cDNA clone (see above) constructed by Leck, 1993 and also provided by Dr Klaus Matthaei.

pBSK(+)$HG_{z\alpha}$ the $HG_{z\alpha}$ cDNA clone (Matsuoka *et al.*, 1990), also provided by Dr Klaus Matthaei.

λ(DASH®II; Appendix 2.2)$MG_{z\alpha}$, phage genomic clones obtained using the partial mouse cDNA fragment (see above; Leck, 1993).

pBSK(+)pgkNeopla (Appendix 2.3), a plasmid containing the pgkNeopla selectable marker, conferring resistance to neomycin, and constructed by Xiao-Wen Wang and provided by Dr Klaus Matthaei.

pBSK(+)pgkThymidine Kinase(TK)pla (Appendix 2.4), a construct containing the pgkTKpla selectable marker, conferring sensitivity to gancyclovir, constructed by XiaoWen Wang and provided by Dr Klaus Matthaei.

Section 2.1.4 Molecular Weight Standards.

20µg of λ phage DNA (48.5kb; NEB) was cut with either *Acc*I or *Hin*dIII in a 400µl volume to obtain the molecular weight DNA standards, λ/*Acc*I (13.070kb, 11.828kb, 6.957kb, 5.581kb, 3.574kb, 2.720kb, 2.180kb, 1.444kb, 0.639kb & 0.499kb) and λ/*Hin*dIII (23.130kb, 9.419kb, 6.557kb, 4.371kb, 2.322kb, 2.028kb, 0.564kb & 0.125kb) respectively. Once completely digested 200µl of 5x Loading Buffer (50mM Tris-HCl pH 8.0, 100mM EDTA, 1% Sarcosyl, 7.5% Ficoll 400, 0.05% Bromophenol Blue & 0.05% Xylene Cyanol) was added to give a final concentration of 0.5µg/15µl. Before electrophoresis each sample was heated (65°C, 15min).

2.1.5 Media and Solutions.

a) Culture Media. Unless otherwise indicated Luria-Bertani (LB) broth and LB agar were used to grow bacterial strains on solid media (1.5% agar) and in suspension (Sambrook *et al.*, 1989). Bacto-yeast extract, tryptone and agar were obtained from Difco Laboratories, whilst ampicillin (Sigma; 50µg/ml in solid media & 100µg/ml in liquid media) was used to select and maintain recombinant bacterial strains.

b) Reagents. The reagents used were of analytical grade and were obtained from: Ajax; Boehringer Mannheim; Bio-Rad; Sigma; Hopkins & Williams; Rhone-Poulenc; Pharmacia; Bethesda Research; and Progen Industries.

Part 2.2 Experimental Protocols.

Section 2.2.1 Preparation of Bacterial Plasmid DNA.

a) Large Scale Alkali Lysis. Large scale preparations of plasmid DNA were performed following the three step alkali lysis method of Garger *et al.*, 1983. The cells from a 1l overnight culture were harvested (6 000xg; 15min at 4°C), resuspended in 24ml of Solution I (50mM Glucose, 10mM EDTA & 25mM Tris-HCl pH8), lysed by adding 55ml of Solution II [0.2M NaOH & 1% Sodium Dodecyl Sulphate (SDS)] and precipitated by adding 28ml of Solution III [5M Potassium Acetate (KOAc) pH4.8]. High molecular weight RNA, chromosomal DNA and protein were then pelleted by centrifugation (20 000xg; 20min at 4°C, Beckman) and the DNA extracted with an equal volume of TE [10mM Tris-HCl pH8.0 & 1mM Ethylenediaminetetraacetic acid (EDTA)]-saturated phenol:chloroform :isoamyl alcohol (25:24:1). The DNA was rescued from the aqueous phase with 0.6 (v/v) 100% propan-2-ol, collected by centrifugation (10 000rpm; IEC Centra M2; 35min, 4°C), washed with 70% ethanol (EtOH), dried and resuspended in 28ml TE. To remove any small molecular weight RNA and contaminating protein, the DNA was purified on a Caesium Chloride (CsCl)/Ethidium Bromide (EtBr) gradient (80 000rpm; 8-12hrs, 22°C) and recovered through a side puncture. The EtBr was then extracted using isoamyl alcohol and the CsCl removed by overnight dialysis against TE.

b) Small Scale Alkali Lysis. A small scale DNA preparation based on the above protocol (Ausubel *et al.*, 1989) was used to obtain plasmid DNA for the rapid analysis of cloned products. Overnight bacterial cultures (1.5ml) were transferred to microfuge tubes, pelleted by centrifugation (45sec), resuspended in 200µl of Solution I, lysed by adding 200µl of freshly prepared Solution II and precipitated with 150µl of Solution III. Each mix was then spun in a bench top microfuge (12 000rpm; 5min), the supernatant removed and the DNA extracted with an equal volume of TE-saturated phenol:chloroform :isoamylalcohol (25:24:1). The DNA was then precipitated with two volumes of 100% EtOH, pelleted, washed with 70% EtOH, dried in a vacuum desiccator and resuspended in 50µl of TE (pH8.0) containing DNase free RNase A (10µg/ml).

c) Large Scale Column Purification. The Qiagen Plasmid Medi® protocol was used to obtain large quantities of highly pure plasmid DNA. The procedure itself is based upon the alkali lysis method (Garger *et al.*, 1983), however, in this instance the DNA is recovered by binding to the 'Qiagen' anion-exchange resin. 25ml of an overnight culture was harvested (6000xg; 15min at 4°C), resuspended in 4ml Buffer P1 (50mM Tris-HCl pH8.0, 10mM EDTA & 100µg/ml RNase A), lysed through the addition of 4ml Buffer P2 (2M NaOH & 1% SDS) and incubated at room temperature for 5min. 4ml of Buffer P3 (3.0M KOAc pH5.5) was then added and this was followed by a further incubation (15min, ice). To remove precipitate the solution was mixed again, spun (20 000xg; 15min, 4°C) and the supernatant taken to a fresh tube. Any suspended material, which could cause problems during column purification was removed carefully and each column equilibrated by applying 4ml Buffer QBT [750mM NaCl, 50mM MOPS pH7.0, 3-(N–morpholino)propanesulfonic acid pK_a7.2), 15% EtOH & 0.15% Triton X-100]. The supernatant was then loaded onto each column and allowed to drain under gravity through the Qiagen tip. To remove residual buffers the columns were washed twice with 10ml Buffer QC (1.0M NaCl, 50mM MOPS pH7.0 & 15% EtOH), the DNA recovered using 5ml Buffer QF (1.25M NaCl, 50mM Tris-HCl pH 8.5 & 15% EtOH), precipitated with 0.7 (v/v) isopropanol and collected by centrifugation (15 000xg; 30min, 4°C). The resulting pellet was washed with 70% EtOH and resuspended in TE.

d) Small Scale Column Purification. The Wizard® Minipreps Purification System (Promega) is adapted from many of the standard miniprep bacterial plasmid DNA protocols that have been used previously (Garger *et al.*, 1983), but differs in that it uses a silica based resin to harvest plasmid DNA following cell lysis and KOAc precipitation. The decision to use this protocol was based upon the need to obtain small quantities of highly pure DNA for use in sequencing and subcloning. Another advantage of using this method is that many DNA preparations can be conducted simultaneously and with a consistent yield, through the use of Promega's Vac-Man® laboratory vacuum manifold. For each preparation 3ml of an overnight bacterial culture was pelleted by centrifugation for 1-2min in a bench top microfuge and resuspended in 200µl of the 'Cell Resuspension Solution' (50mM Tris-HCl pH7.5, 10mM EDTA, 100µg/ml RNaseA). The cells were

then lysed by adding 200µl of the 'Cell Lysis Solution' (0.2M NaOH, 1% SDS) and the protein precipitation by adding 200µl of the 'Neutralisation Solution' (1.32M KOAc pH4.8). After spinning the tubes at top speed in a microfuge for 5min the supernatant/'Wizard' DNA purification resin (1ml) mix was passed through the 'Wizard mini-column', and the solution discarded. The DNA-bound mini column was then washed with 2ml of the 'Column Wash Solution' (200mM NaCl, 20mM Tris-HCl pH7.5, 5mM EDTA, dilute 1:1 with 95% EtOH), removed from the syringe, spun at top speed for 20 seconds and the DNA eluted from the column with 50µl of TE.

Section 2.2.2 Preparation of λ Phage DNA.

The stored lysate from each of the λ phage mouse genomic clones (Part 3.2, p65), diluted to 10^{-8} plaque forming units (pfu) per 10µl was used to infect 200µl of an overnight culture of *E. coli* XL1-Blue containing 25µl 10mM $CaCl_2$ and 25µl 10mM $MgCl_2$. This was left to incubate for 20min at 37°C, mixed with 3ml of Top Agarose (0.7% agarose, prewarmed to 55°C) and poured onto an LB agarose plate. Following overnight incubation at 37°C, a single phage plaque was picked and added to 100µl of phage buffer (20mM Tris-HCl pH7.4; 100mM NaCl; 10mM $MgSO_4$ adjust to pH7.4). The previous day a single colony of *E. coli* XL1-Blue, was used to inoculate 3ml of LB containing 30µl 1M $MgSO_4$ and 3µl 20% Maltose. 500µl of this culture was added to the 100µl of diluted phage and the mix incubated for 20min at 37°C. For each preparation of phage DNA the prepared phage culture was used to inoculate 100ml LB, 1ml 1M $MgSO_4$, prewarmed to 37°C in a 250ml flask. This liquid culture was then incubated (37°C, 3-6hrs) and the OD monitored every 30min or until complete lysis had occurred. After which 0.5ml of chloroform was added with mixing. The culture was then centrifugated (8 500rpm; 10min, 6°C), the supernatant transferred to a clean flask and RNaseA and DNase added to a final concentration of 100µg/ml (30min, 37°C). To precipitate the phage one volume of phage precipitation solution (20% PEG 8 000 & 2M NaCl) made up in SM Buffer (1l: 8g NaCl, 2.8g $MgSO_4$, 6.1g Tris pH7.5; & 0.1g gelatin) was added, the solution incubated (1hr, ice), centrifugated (12 000rpm; 10min, 4°C) and the resulting pellet resuspended in 6ml of L3 buffer (100mM Tris-HCl, 100mM NaCl, & 25mM EDTA pH7.5), 2ml of 10% SDS. This was followed by a further incubation period (70°C,

20min), the λ phage DNA extracted with an equal volume of TE-saturated phenol:chloroform:isoamyalcohol (25:24:1) and the solution spun (4 000rpm). This was repeated twice, the DNA precipitated with 1/10th (v/v) 3M NaOAc pH7.0, equal volume isopropyl alcohol, at room temperature for 20min, the pellet washed with 70% EtOH and dissolved in 500µl TE.

Section 2.2.3 Preparation of Mouse Cerebellum mRNA.

a) Extraction of Total RNA from Mouse Cerebellum. The method used is adapted from the RNA isolation protocol of Chomczynski and Sacchi, 1987. Adult mice (BALB/c, 20-30g) of either sex were killed with an overdose of ether. The skin around the head of the animal was removed and the skull cut open through the temporal ridges. To remove the brain, cuts were made at the orbitals, optic nerves and spinal cord. The cerebellum was then cut out, weighed and placed in a homogeniser containing 1ml of Trisolv® (Biotecx) per 50-100mg of tissue, homogenised and allowed to sit at room temperature for 5min to allow dissociation of nucleoprotein complexes. The tissue was then transferred to a new tube, 0.2ml of chloroform added and vortexed for 15secs. After a further 2-3min the homogenate was centrifugated (12 000xg, 15min, 4°C) and the aqueous layer removed. Note that the same method was used to prepare RNA from the mouse liver, kidneys, lung, spleen, whole brain, lungs and other tissues.

b) RNA Precipitation. the aqueous layer from the above RNA extraction step was transferred to a fresh tube, mixed with isopropanol (0.5ml/1ml of Trisolv®) and stored at room temperature for 5-10min at 4°C. The RNA was then pelleted by centrifugation (12 000xg, 10min, 4°C), and washed twice with 75% EtOH. At the end of the procedure the RNA was then left as a pellet or resuspended in 20-50µl of diethylpyrocarbonate (DEPC) treated-RNase free doubly distilled (dd) H_2O and the yield calculated.

c) Column Purification of mRNA. Whole mouse cerebellum RNA was obtained following the procedure described above and run through oligonucleotide dT cellulose columns (Pharmacia) to specifically isolate the mRNA. Each column was first inverted several times to resuspend the oligonucleotide dT-cellulose, washed with storage buffer and then with 1ml of High Salt Buffer which was allowed to drain through the column under

gravity. The RNA was prepared by resuspending approximately 1.25mg in 1ml of Elution Buffer (TE, in 0.1% DEPC treated ddH_2O), which was then heated at 65°C for 5min, transferred to ice and mixed with 0.2ml of Sample Buffer. The elution buffer was then prewarmed to 65°C, applied to the top of the column bed and allowed to soak under gravity. The column was then spun (350xg; 2min), 0.25ml of High Salt buffer added and spun again (350xg for 2min). This wash step was repeated once more using 0.25ml High Salt Buffer and another three times with 0.25ml of Low Salt Buffer. The RNA was then eluted from the column using 0.25ml aliquots of prewarmed (65°C) Elution Buffer (350xg, 2min). After each spin the eluate was added to 100µl of Sample Buffer, 10µl of Glycogen Solution and 2.5ml of EtOH mix. This solution was then chilled at -20°C for 2hrs and spun (4°C, 12 000rpm) to recover the mRNA which was stored at -70°C until needed. The yield was then calculated using spectrophotometric analysis (Section 2.2.5, p37).

Section 2.2.4 Preparation of Oligonucleotides.

a) Introduction. The desired oligonucleotide (Table 2.1) was prepared by the Biomolecular Resource Facility at the John Curtin School of Medical Research, Australian National University (ANU). The primer would arrive either as lyophilised powder or in ammonium hydroxide. In either case the following protocols were used to further purify each sample.

b) n-Butanol Precipitation from Ammonium Hydroxide. In this procedure 1ml of n-butanol was added to 100µl of the ammonium hydroxide solution. This was vortexed for 15 to 20 seconds and spun at high speed for several minutes. The pelleted oligonucleotide was washed with 70% EtOH, dried and resuspended in 100µl ddH_2O.

c) Diethyl Ether Precipitation from Acetic Acid. About 200nmoles of the lypholised oligonucleotide was first redissolved in 10µl of ddH_2O, mixed and 40µl of glacial acetic acid added in a step wise manner until the oligonucleotide was completely dissolved. 1ml of diethyl ether was then added and the solution was left to stand (30min). The oligonucleotide was then pelleted, washed with diethyl ether and dissolved in ddH_2O.

Table 2.1 Oligonucleotides Utilised.

*T_H (Hybridisation Temperature)=T_M(Melting Temperature)-5°C

T_M=2x(nA+nT)+4x(nG+nC) and n=number of A's, number of C's etc.

	5' Oligonucleotide 3'	*T_H
1BF	GCCTGCAAGGAGTACAAGCCC	63°C
4BF	CCCGAGCTGCTGGGTGTCATG	65°C
3R	GCCAATGTACTTGAGATTGTTCTG	63°C
anti-1BF	GGGCTTGTACTCCTTGCAG	55°C
anti-4BF	CATGACACCCAGCAGCTCG	57°C
4CF	CCTGAGCTGCTGGGTGTCATG	65°C
anti-4CF	CATGAGACCCAGCAGCTCA	52°C
Al-1	CATCCGCAACCCTACTCAGAGATT	67°C
Al-2	TTTGCTATAAACGGAGGCTCTAGG	65°C
NeoP5	GCCTGAAGAACGAGATCAGCAGCC	71°C
NeoP6	GCCAAGTTCTAATTCCATCAGAAGC	67°C
Universal	TGACCGGCAGCAAAATG	47°C
Reverse	GGAAACAGCTATGACCATG	49°C
prom1	CGTGCTACTTCCATTTGTCACGTC	67°C
prom2	GGTGGATGTGGAATGTGTGCGA	63°C
mG_ZG_F	ATGGGATGTCGGCAAAGCTCAGA	65°C
mG_ZG_F(NotI)	GCGGCCGCATGGGATGTCGGCAAAGCTCAGA	65°C
mG_ZG_R	TACTCCTTGCAGGCGTCCAGGTT	67°C
NOTIPA	CAATTCGCGGCCGCTTTTTTTTTTTTTTT	NA
NOTPCR	CTCAATTCGCGGCCGCTTTT	62°C

Section 2.2.5 Spectrophotometric Analysis.

Following the preparation of RNA, single stranded (ss)DNA double stranded (ds) DNA, or oligonucleotide, 5µl of each sample was diluted in 1000µl of ddH_2O and the optical density at 260nm (OD_{260}) used to obtain the concentration (C; Sambrook *et al.*, 1989).

OD_{260}=Absorbance at 260nm (A_{260})x1000/5.

OD_{260}1=50µg/ml therefore C(dsDNA)=50xOD_{260}µg/ml

=33µg/ml therefore C(ssDNA)=33xOD_{260}µg/ml

=40µg/ml therefore C(RNA)=40xOD_{260}µg/ml for

The following formula was used to calculate the concentration of each oligonucleotide:

C=(A_{260} x 100 x dilution)/[(1.54xnA)+(0.75xnC)+(1.17xnG)+(0.92xnT)]

Part 2.3 Recombinant DNA Techniques.

Section 2.3.1 Restriction Endonuclease Digests.

Each restriction digest was carried out in the appropriate 1xNEB buffer for that enzyme. These include: NEBuffer 1 (10mM Tris Propane-HCl, 10mM $MgCl_2$ & 1mM dithiothreitol (DTT) pH7.0); NEBuffer 2 (10mM Tris-HCl, 10mM $MgCl_2$, 50mM NaCl & 1mM DTT pH7.9); NEBuffer 3 (50mM Tris-HCl, 10mM $MgCl_2$, 100mM NaCl & 1mM DTT pH7.9); NEBuffer 4 (20mM Tris-acetate, 10mM MgAc, 50mM KOAc & 1mM DTT pH7.9); *Eco*RI Buffer (50mM NaCl, 100mM Tris-HCl, 10mM $MgCl_2$, 0.025% Triton X-100 pH7.5); and *Bam*HI Buffer (150mM NaCl, 10mM Tris-HCl, 10mM $MgCl_2$, 1mM DTT pH 7.9). For most digests a mix containing 20µl (1µg; 0.05µg/ml) of plasmid DNA, 2µl of 10x NEBuffer, 2µl of 10x acetylated-Bovine Serum Albumin (BSA) and 2U of enzyme was prepared and incubated at 37°C for 60min.

Section 2.3.2 Analytical and Preparative Separation of DNA Fragments.

In order to determine the success of a restriction digest, polymerase chain reaction (PCR) or to estimate DNA concentration and quality, electrophoresis was carried out at room temperature on horizontal 1% agarose gels. The gels were prepared by dissolving agarose in TAE (40mM Tris-acetate & 2mM EDTA pH8.0). Ethidium bromide (EtBr) was then added to both the gel and buffer (TAE) to a final concentration of 0.5µg/ml. The gels were submerged in either a Pharmacia or Biorad Mini-Submarine® electrophoresis tank and electrophoresis was conducted at 80V for 1-2hrs. Following separation by electrophoresis the DNA was visualised under ultraviolet (UV) light and photographed.

Section 2.3.3 Gel Purification of Restricted DNA Fragments.

a) Glass Milk Extraction. This protocol was adapted from the Gene Clean® system (Bio 101, Bresatec) which utilises Glass Milk to bind DNA. Following electrophoretic separation the desired band was cut from the agarose using a scalpel blade, submerged in 3xNaI and incubated (5min, 55°C). Once the agarose had completely melted 5µl of glass milk was added. This was followed by a further incubation period on ice (5min), the

solution spun at top speed (12 000rpm, 5sec) and the supernatant removed. The glass milk/bound DNA was then washed in 300µl of the 'New Wash' solution and repelleted (12 000xg, 5sec). This was repeated twice more and after the final wash the pellet was resuspended in 6µl of TE, incubated for 2min at 55°C, spun at top speed (12 000xg, 30sec) and the supernatant removed. This step was repeated and the yield calculated.

b) Squeeze/Freeze Method. In each instance the DNA band cut from the gel was wrapped in wax paper and stored at -20°C for 1-2hrs. The frozen block of agarose was then 'squeezed/thawed' between two pieces of parafilm and the buffer collected in a tube. The DNA was then precipitated (2x100% EtOH, 1/10th volume 3M NH_4OAc pH 5.3), pelleted (12 000xg, 30min), washed with 70% EtOH and resuspended in 20-30µl TE.

Section 2.3.4 Blunt Ending DNA fragments.

The Klenow fragment of T_4 DNA polymerase is a DNA dependent DNA polymerase which, although it retains its 3' to 5' exonuclease has lost the 5' to 3' exonuclease activity (Sambrook *et al.*, 1989). This means that the enzyme can be used to catalyse the 'filling in' of 5' overhangs left following enzyme restriction. Each reaction was carried out in 1xKlenow Buffer (10mM Tris-HCl pH7.5, 5mM $MgCl_2$ & 7.5mM DTT; NEB) supplemented with 33µM of each deoxynucleotide triphosphate (dNTP) for 10min at 37°C and the enzyme deactivated (70°C, 5-10min).

Section 2.3.5 Dephosphorylation of Linearised Vectors.

The dephosphorylation reaction removes 5' phosphate groups from the ends of linearised plasmid and phage vector DNA, thus preventing vector self ligation (Sambrook *et al.*, 1989). Each reaction was conducted in either one of the four NEB restriction buffers (Section 2.3.1, p38) and catalysed by 2U of the Calf Intestinal Phosphatase (CIP). Following a 30min incubation at 37°C, the mix was transferred to a 50°C water bath and left for another 30min. This 50°C step is especially important when 'phosphatasing' blunt ends. The enzyme was then deactivated by phenol:chloroform extraction, the plasmid DNA precipitated by the addition of two volumes 100% EtOH, 1/10th volume 3M NH_4OAc pH5.3, centrifugated and the pellet resuspended in 20µl ddH_2O.

Section 2.3.6 Ligation of DNA Fragments.

Ligations were conducted to stably clone restricted DNA fragments. In each instance the ligation mix consisted of DNA/vector in a 2:1 ratio, 1xT_4 DNA Ligase Buffer (50mM Tris-HCl pH 7.8, 10mM $MgCl_2$, 10mM DTT, 1mM dATP, 25mg/ml of Bovine Serum Albumin), 10U of T_4 DNA Ligase (NEB) and ddH_2O in a 20µl reaction volume. The ligation was then incubated in a water bath (Blunt End Cloning, overnight 16°C; Sticky or Sticky/Blunt Cloning, 2-3hrs room temperature). The controls used in each case include: (i) vector and T_4 DNA ligase but no insert, to indicate the degree of vector religation; and (ii) vector alone to indicate the degree of digestion.

Section 2.3.7 Transformation and Selection of Bacterial Clones.

a) Preparation of Chemically Competent Cells. Chemically competent *E. coli* strains were prepared by diluting (1:100) an overnight culture of the desired strain in LB broth and grown to an OD_{600}=0.6 in a 1l conical flask. This culture was then harvested in two 50mL polypropylene tubes, chilled on ice for 10-15min and spun (1000xg; 12-15min, 4°C). The resultant pellet was then resuspended in cold 16.7ml RF1 solution (100mM RbCl, 50mM $MnCl_2.4H_2O$, 30mM 1M KOAc pH7.5, 10mM $CaCl_2.2H_2O$ & 15% Glycerol. Adjusted to pH5.8 with 0.2M Acetic Acid. Filter sterilise), repelleted (1000xg; 12-15min, 4°C) and resuspended in 4ml RF2 solution (10mM RbCl, 75mM $CaCl_2.2H_2O$, 10mM 0.5M MOPS pH6.8 & Glycerol 15%. Adjust to pH6.8 with NaOH. Filter sterilise). After this wash the cells were kept on ice for 15min and stored (-70°C).

b) Transformation. For each transformation 20µl of ligation mix was added to a single 200µL aliquot of chemically competent bacterial cells, thawed on ice. After 30min on ice this transformation mix was then heat shocked (42°C, 90sec), allowed to recover in 1ml of LB (37°C, 60min), plated onto LB agar containing appropriate antibiotic selection media (e.g. ampicillin 50µg/ml) and incubated at 37°C, overnight (Brown *et al.*, 1979).

c) Preparation of Electrocompetent Cells. A bacterial culture of the desired *E. coli* strain was grown overnight (37°C) in 10ml of SOB media (4% Tryptone, 1% Yeast Extract, 0.02M NaCl & 0.005M KCl, autoclave then add $MgSO_4$ to final concentration 0.04M). This starting culture was then diluted in 1l of LB broth and grown with vigorous shaking

at 37°C to OD_{600}0.5-1. When this density was reached the cells were harvested by centrifugation (4000xg; 15min, 4°C) and resuspended in an equal volume of cold 10% glycerol. This washing step was repeated several times with 500ml, 20ml and 3ml of the 10% glycerol solution. Following this final wash the cells were aliquoted, snap frozen in liquid nitrogen and stored at -70°C.

d) Electroporation. Prior to electroporation electro competent cells were allowed to thawed on ice. A 40µL aliquot was then combined with 1µl of the ligation mix and transferred to an ice cold micro-cuvette (Biorad®) and electroporated (25µF, 2.5kV and 200 ohms) with the Biorad® Gene Pulser. 1ml of SOC broth (SOB+0.02M Glucose) was then added and the cells allowed to recover at 37°C for 60min. Several dilutions of this mini-culture were then plated onto LB agar bacterial plates, containing the appropriate antibiotic and incubated overnight at 37°C.

Section 2.3.8 Screening Cloned Inserts.

a) Blue/White Selection System. 25µl X-gal [5-bromo-4-chloro-3-indolyl-β-D galactose; 2% dimethylfluoride (DMF)] and 15µl IPTG (100mM) was spread out evenly and allowed to dry on each LB agar plate prior to spreading the transformation mix (see above). Present on certain cloning vectors the *lacZ* gene codes for the expression of enzyme β-galactosidase which is induced by IPTG and uses X-gal as a substrate to produce a blue colouration in those cells which contain the vector. If the expression of the gene is interrupted by a cloned insert the fusion protein which is produced will be incapable of utilising X-gal. Those cells containing vectors with cloned inserts will, therefore, appear white (Sambrook *et al.*, 1989). This form of screening, however, gives no indication of the size of the insert and false positives are common due to random deletions which inactivate the *lacZ* gene.

b) Colony Cracking. In most instances and whether or not blue/white selection was used all transformants were subjected to analysis by colony 'cracking'. A well isolated colony was picked with a sterile toothpick, replicated onto a fresh agar plate and resuspended into 25µl of the 'cracking solution' (0.01% Bromocresol green, 0.005M EDTA, 0.05M NaOH, 0.5% SDS & 10% Glycerol). This was repeated as many times as necessary and the

replica plate placed in a 37°C incubator, so that the transferred cells could be used once positive clones were identified. All of the samples were then incubated at 65°C for 30min, allowed to cool to room temperature, and subjected to agarose (1%) gel electrophoresis. Positive clones were easily identified by their increased size when compared with uncut vector and due to the addition of the cloned insert.

Section 2.3.9 The Reverse Transcriptase (RT) Reaction.

a) Oligonucleotide dT Primers. For separate non-specific first strand synthesis of mRNA, an 18mer oligonucleotide dT primer was used prior to PCR amplification of a specific cDNA product. The RT mix was made up with 5x AMV RT Buffer, 500µM dNTP mix, 10mM DTT and 0.5µg primer/µg total RNA. The reaction was conducted in a sterile 0.5mL eppendorf tube, incubated (42°C, 1hr) and the enzyme heat killed (65°C, 15min).

b) The Promega Riboclone® System. The Riboclone® System was used for first strand synthesis of mRNA and was used in an attempt to clone a full length $MG_{z\alpha}$ cDNA clone. 0.5µg of the NOTIPA (Table 2.1) was added for every 1µg of purified mRNA in a total volume of 15µl with ddH_2O. This mix was then heated to 70°C for 5min and allowed to cool to room temperature. To the annealed primer and template was added 5µl first strand 5x AMV Buffer (Promega), 25U rRNasin® ribonuclease inhibitor, 2.5µl 40mM sodium pyrophosphate, 15U/µg RNA AMV RT and ddH_2O to a volume of 25µl. Each of these components (except the enzyme which was added last) were preheated to 42°C for 5min to prevent the sodium pyrophosphate from precipitating. The reaction was then incubated (42°C, 1hr) and 50mM EDTA added. This RT mix was stored at -20°C, until needed.

Section 2.3.10 The Polymerase Chain Reaction (PCR).

Each 20µl PCR reaction was made up in 1xPCR buffer (GeneAmp® PCR Reagent Kit; 10mM Tris-HCl pH 8.3, 50mM KCl, 1.5mM $MgCl_2$, 0.001% gelatin), 200µM of each dNTP, 1U Supertaq® DNA polymerase and 5pmol of each primer. The PCR reaction mixtures were pippetted into positive displacement pipette tips, using a Cycle Prep® automatic pippetor. The PCR reaction was then carried out in a Corbett Research fast capillary thermal sequencer. The cycling conditions for each reaction are shown in each of the following chapters. The PCR cycling conditions used to amplify the $MG_{z\alpha}P$ cDNA

fragment were as follows: DNA denaturation (95°C, 5min), annealing temperature (T_A; 63°C, 4BF/3R), annealing time (AT; 5sec), primer extension temperature (T_E; 72°C) and primer extension time (ET; 30sec). This cycle was repeated 35 times (35x) and followed by a final primer extension (72°C) period of 3min. In each instance 2µL of 5x Loading Buffer (Part 2.1, p30) was added and each sample heated for 15min at 65°C, prior to agarose gel electrophoresis.

Section 2.3.11 The One-step RT-PCR Reaction.

The RT-PCR protocol developed by Sellner *et al.*, 1992 was used to isolate the partial $MG_{z\alpha}$ cDNA clone (Leck, 1993), and in subsequent attempts to obtain a full length cDNA $MG_{z\alpha}$ clone. Each 20µl reaction contained mouse cerebellum total RNA (or mimic RNA control; Chapter 4, p98), 200µM of each dNTP, 1x PCR (GeneAmp®) or RT-PCR Buffer (Leck, 1993; 67mM Tris pH 8.3, 0.5mM DTT, 50mM KCl, 0.1% Triton X-100, 6µM EDTA & 2mM $MgCl_2$), 0.5U of AMV RT, 5pmol of forward primer (4BF) and 5pmol of reverse primer (3R) to primer the RT reaction. The reactions were conducted in positive displacement pipette tips (see above), using the following cycling conditions for this example. The RT reaction (42°C, 59min), RT inactivation (95°C, 5min). PCR amplification: DNA denaturation (95°C, 5sec) primer annealing (T_A, 63°C; AT, 5sec), primer extension (T_E, 72°C; ET, 2min). This was repeated 35 times and a second step of primer extension added (72°C, 7min). The enzyme was then inactivated (99°C, 10min) and the solution stored at 4°C. Note that prior to agarose (1%) gel electrophoresis each sample was heated (65°C, 15min).

Part 2.4 Hybridisation Protocols.

Section 2.4.1 Preparation of Radiolabelled Probes.

a) End Labelling. All oligonucleotide probes were radioactively endlabelled (Sambrook *et al.*, 1989). The concentration of each primer was first carefully determined by spectrophotometric analysis and diluted to 5pmol/µl. 0.4pmol (0.8µl) of the oligonucleotide was then diluted in 19.2µl ddH_2O, to which was added 3µl 10xKinase Buffer (70mM Tris-HCl pH7.6, 10mM $MgCl_2$ & 5mM DTT; NEB), 3µl acetylated-BSA (10µg/ml, NEB), 1µl 0.1M DTT; 1µl Spermidine, 1µl γ^{32}P-ATP (1 000Ci/mmol) and 1µl T_4 Kinase (NEB). The solution was then mixed, 1µl removed as a control and incubated (15min, 37°C). At the end of this time 3µl of 10xStop buffer (0.125M EDTA & 5% SDS) was added and 1µl of the labelled probe removed for chromatographic analysis.

b) Random Priming. Larger fragments of DNA were radiolabelled by random priming (Amersham). To 2.5µl of DNA (50ng) was added 5µl of Primer Solution and 12.5µl of ddH_2O. The solution was then heated (95°C, 5min) to denature the dsDNA and the primers allowed to bind at room temperature. The labelling reaction was at 37°C for 30min in a 50µl volume which contained 5µl of 10x Labelling Buffer, 4µl each of cold nucleotides (dTTP, dGTP & dCTP), 2µl of α^{32}P-dATP (10µCi/µl), 5µl BSA, 9µl ddH_2O and 1U Klenow. Labelled probe was then precipitated by adding 5.5µL 3M NaOAc pH5.3, 120µl 100% EtOH and 5µl (100µg/ml) of ssDNA. The mix was then spun (12 000rpm; 20min) and the pellet resuspended in 100µl ddH_2O.

Section 2.4.2 Analysis of Radiolabelled Probes.

a) Thin Layer Chromatography (TLC). After preparing the probe 1µl of the control and 1µl of the reaction were spotted onto PEI (polyethylene imine) paper (Merck) and lowered into PEI buffer (0.75M NaH_2PO_4 & 0.75M H_3PO_4 pH3.5) for 20min. The paper was placed under X-ray film (Xerox/Fuji; room temperature, 20min). The degree of incorporation was determined by visually comparing the signal of the unincorporated radiolabelled dNTPs with the amount released by the labelled probe (Figure 3.6).

b) Scintillation Counting. To obtain a more definitive measure of radioactivity scintillation counting was used. 1μL of labelled purified probe was placed in an eppendorf tube and the counts/min/μg measured using a Rack Beta Liquid Scintillation Counter 1217.

Section 2.4.3 DNA Transfer and Hybridisation.

a) Southern Transfer. The blotting protocol used was adapted from an alkaline transfer method that utilises the capillary action generated by layers of folded paper (Reed & Mann, 1985). Two large pieces of 3MM Whatman paper were soaked in 0.4M NaOH and placed on a tray above a dish containing transfer solution (0.01M NaOH). The depurinated (0.25M HCl, 10min) agarose gel was then placed in water to remove excess HCl and allowed to soak briefly in 0.4M NaOH before being layered on top of the Whatman paper. The positively charged nylon membrane (PCNM; Hybond N^+) which was also washed in 0.4M NaOH, carefully placed on top of the gel and the position of the wells marked. It was not necessary to introduce a separate denaturing step prior to blotting as this process would occur during transfer. Several layers (5-10) of 3MM Whatman paper were then layered above the PCNM membrane, and were placed many folded sheets of paper (15cm) surmounted by a weight, to keep it all in place. To increase the efficiency of transfer wet sheets were replaced with dry ones after 2hrs. The transfer was allowed to continue for up to another 2hrs before the membrane was removed.

b) Alkaline DNA Dot/Slot Blotting. Vacuum blotting was used to quickly transfer λ phage DNA to positively charged membranes (Hybond N+), for hybridisation with radiolabelled 3R. This procedure used to strip coat protein from the phage plaques, could also be applied to: (i) purified or crude DNA (<5μg); (ii) whole soft tissue (e.g. liver, <0.5mg); (iii) whole blood (<10μl); cultured cells; or (iv) sperm (Reed & Matthaei, 1990). The dot/blot apparatus was assembled with a sheet of PCNM. Each of the samples, prepared by heating (95°C; 0.4M NaOH), were added to the wells in batches of eight (0.5ml). The vacuum was then applied to transfer the prepared material to the membrane. Immediately following this the membrane was washed (2x PE & 0.1% SDS), dried and stored at 4°C, until used.

c) DNA Probes. Prior to hybridisation with ramdomly primed radiolabelled DNA fragments the membranes were incubated for 1hr at 65°C in 10ml of prehybridisation solution containing 0.5% SDS, 6x SSC (17% NaCl; 8.82% Sodium citrate pH7.0), 0.01M EDTA, 5x Denhardts (0.5% Ficoll, 0.1% Polyvinylpyrrolidone, 0.1% fraction V BSA) and 100µg/ml denatured (95°C, 5min) salmon sperm DNA (Sambrook *et al.*, 1989). The radiolabelled probe (about $2x10^5$ cpm/µl) was then denatured (95°C, 5min) added to the prehybridisation solution and the membrane left to incubate (4-5hrs, 65°C) in a Hybaid® oven. At the end of this time the filters were washed twice with shaking at room temperature (20min; 2xSSC & 0.1% SDS). This was followed by further high stringency washes (65°C; 0.1xSSC & 0.1% SDS). The membrane was then exposed to X-ray film for up to a week at -70°C.

d) Oligonucleotide Probes. When using an oligonucleotide probe the membranes were incubated for 1-2hrs in 20mL of the prehybridisation solution [5x PE (0.133M Sodium Phosphate pH6.9 & 0.001M EDTA), 7% SDS & 1% BSA] at the T_H. At the end of this time 10ml of the solution was removed, end-labelled probe added and the membrane left to incubate for 4-5hrs at the T_H. A set of washing steps were then conducted to remove any unbound or non-specifically attached probe. The membrane was first washed in 5x SSC (14.17% NaCl & 7.35% Sodium Citrate), 0.1% SDS at room temperature for 15min. This was followed by a temperature wash at the T_H in 5x SSC, 1% SDS for a further 15min and a final washing step at room temperature in 2x SSC and 0.1% SDS for 10min. The membrane was then exposed to X-ray film for up to a week at -70°C.

e) Stripping the Radiolabelled Probes. In a number of instances the same membrane was hybridised with different probes. Before each procedure the previous probe was removed, either through boiling the membrane in 0.1% SDS, and allowing it to cool to room temperature (Sambrook *et al.*, 1989) or through the use of a gentle stipping solution containing 110ml Formamide, 20ml 20xSSPE, 10ml 20% SDS, made up to 200ml with ddH_2O. Gentle stripping was conducted within a Hybaid® oven at 65°C for 15 to 30min, or until the label was removed. Note that in either case the stripped membrane was exposed to X-ray film overnight, to confirm that no bound label remained.

Section 2.4.4 RNA Transfer and Hybridisation.

a) Preparation and Electrophoretic Separation of RNA. Prior to use RNA samples stored under ethanol were centrifuged, rinsed, dried and resuspended in a small volume of TE plus 2mM DTT and 1U/µl RNasin RNase inhibitor (Promega). Samples were prepared for electrophoresis by mixing 10µg of RNA with 2µl of 10xMOPS buffer (0.2M MOPS, 0.05M NaOAc pH7.0, 0.01M disodium EDTA & 0.1% DEPC), 3.3µl 37% formaldehyde and ddH_2O to 20µl. This mix was heated at 55°C for 15min and 2µl of 10xRNA sample buffer (Loading buffer made up in DEPC treated ddH_2O) added. Electrophoresis of RNA was carried up in a denaturing gel consisting of 0.188g of iodoacetamide, 0.3ml of EtOH, 1% agarose, 10ml 10x MOPS buffer, 2ml 37% formaldehyde solution, 5ml EtBr (1mg/ml) and 86ml ddH_2O. The gel was run at 80V for approximately 60min using 1xMOPS buffer as the running buffer.

b) Northern Transfer. Northern transfer of RNA was conducted in a similar manner to Southern DNA blotting, however, in this instance 1xMOPS buffer was used as the RNA transfer buffer, whilst the denaturing and depurinating steps were excluded. The RNA was fixed to the membrane using UV illumination.

c) Hybridisation of RNA Filters. Each RNA-bound membrane was incubated (42°C, overnight) in Hybaid® bottles with 10ml of prehybridisation solution (5xSSC, 5xDenhardts, 0.5% SDS & 50% Formamide). To this was added 100µl 10mg/ml sheared salmon sperm DNA, boiled for 10min prior to incubation. Following this period the desired probe (see above) was boiled and added directly to the solution. This second hybridisation step was left for 8hrs and the membrane washed: (i) 5-10min, 2xSSC, 0.1% SDS, room temperature; (ii) 15min, 2xSSC, 0.1% SDS, 55°C; (iii) 15min, 1xSSC, 0.1% SDS, 55°C; and (iv) 15min, 0.5xSSC, 0.1% SDS, 55°C, if required. The membrane was then placed under X-ray film (overnight to one week; -70°C).

Part 2.5 The Generation of Deletion Clones.

Section 2.5.1 Introduction.

The Erase a base® sequencing protocol is a rapid and relatively efficient means of generating overlapping sequences, from a single large DNA fragment (Promega). The procedure itself involves the use of the DNase *Exo*III which cuts dsDNA from unprotected 5' ends (not 3' or α-phosphorothioate protected DNA ends) at a defined rate, creating a number of deletion fragments which can be separately recircularised, cloned and sequenced (Figure 2.1A).

Section 2.5.2 Protection of 5' Protruding Ends.

Approximately 5μg of the designated clone was digested with the enzyme chosen to generate the 'primer protecting' site (e.g. *Not*I; Table 2.2). If Klenow was active in this restriction buffer, the 'fill in reaction' (of the 5' overhangs with α-phosphorothioate nucleotide's) was performed directly. However in the cases that it was not, the DNA was first extracted. The volume of the digestion mix was brought to 100μl with ddH_2O and added to an equal volume of TE-saturated phenol:chloroform:isoamylalcohol (25:24:1). The mixture was then vortexed and the two phases separated by centrifugation (12 000xg, 5 min). The aqueous layer was then transferred to a fresh tube, to which was added 1/10th volume of 2M NaCl and 2x 100% EtOH. This mix was then left at -20°C for 10min, centrifugated (12 000xg, 5min) and the DNA pellet washed with 70% EtOH, dried and resuspended in 50-100μl of 1xKlenow Buffer (Promega).

Section 2.5.3 *Exo*III Deletion, Ligation and Transformation Procedures.

The following example given is based upon using 5μg of the 4kb *Kpn*I clone 8K (Section 3.2.6, p76). The protected linearised clone was cut with *Eco*RI to expose one end, precipitated and resuspended in 50μl of 1x*Exo*III buffer (50mM Tris-HCl pH8.0, 5mM $MgCl_2$ & 10mM 2-mercaptoethanol). Meanwhile 7.5μl of S1 nuclease mix [25 time points: 172μl ddH_2O, 27μL S17.4xbuffer (0.3M KOAc, 2.5M NaCl, 10mM $ZnSo_4$ & 50% Glycerol) & 60U *Exo*III] was aliquoted into twenty small tubes and left on ice. The

Table 2.2 Restriction Enzyme Recognition Sites.

(Taken from the Promega Catalogue)

Restriction Enzymes That Generate *Exo*III Resistant 3' Overhangs		Restriction Enzymes That Generate 5' Overhangs or Blunt Ends	
Enzyme	Recognition Sequence	Enzyme	Recognition Sequence
*Aat*II	5'...GACGTC...3' 3'...CTGCAG...5'	*Bam*HI	5'...GGATCC...3' 3'...CCTAGG...5'
*Ban*II	5'...GPuGCPyC...3' 3'...CPyCGPuG...5'	*Cla*I	5'...ATCGAT...3' 3'...TAGCTA...5'
*Bgl*I	5'...GCCNNNNNGGC...3' 3'...CGGNNNNNCCG...5'	*Csp*451	5'...TTCGAA...3' 3'...AAGCTT...5'
*Bst*XI	5'...CCANNNNNNTGG...3' 3'...GGTNNNNNNACC...5'	*Eco*RI	5'...GAATTC...3' 3'...CTTAAG...5'
*Hae*II	5'...PuGCGCPy...3' 3'...PyCGCGPu...5'	*Eco*RV	5'...GATATC...3' 3'...CTATAG...5'
*Hha*I	5'...GCGC...3' 3'...CGCG...5'	*Hind*III	5'...AAGCTT...3' 3'...TTCGAA...5'
*Kpn*I	5'...GGTACC...3' 3'...CCATGG...5'	*Nco*I	5'...CCATGG...3' 3'...GGTACC...5'
*Nsi*I	5'...ATGCAT...3' 3'...TACGTA...5'	*Nde*I	5'...CATATG...3' 3'...GTATAC...5'
*Pvu*I	5'...CGATCG...3' 3'...GCTAGC...5'	*Not*I	5'...GCGGCCGC...3' 3'...CGCCGGCG...5'
*Sac*I	5'...GAGCTC...3' 3'...CTCGAG...5'	*Sal*I	5'...GTCGAC...3' 3'...CAGCTG...5'
*Sph*I	5'...GCATGC...3' 3'...CGTACG...5'	*Sma*I	5'...CCCGGG...3' 3'...GGGCCC...5'
		*Spe*I	5'...ACTAGT...3' 3'...TGATCA...5'
		*Xba*I	5'...TCTAGA...3' 3'...GAGCTC...5'
		*Xho*I	5'...CTCGAG...3' 3'...GAGCTC...5'

(*) Although *Apa*I, *Pst*I and *Sac*II generate 3' overhangs, these ends are not protected from *Exo*III (see below)

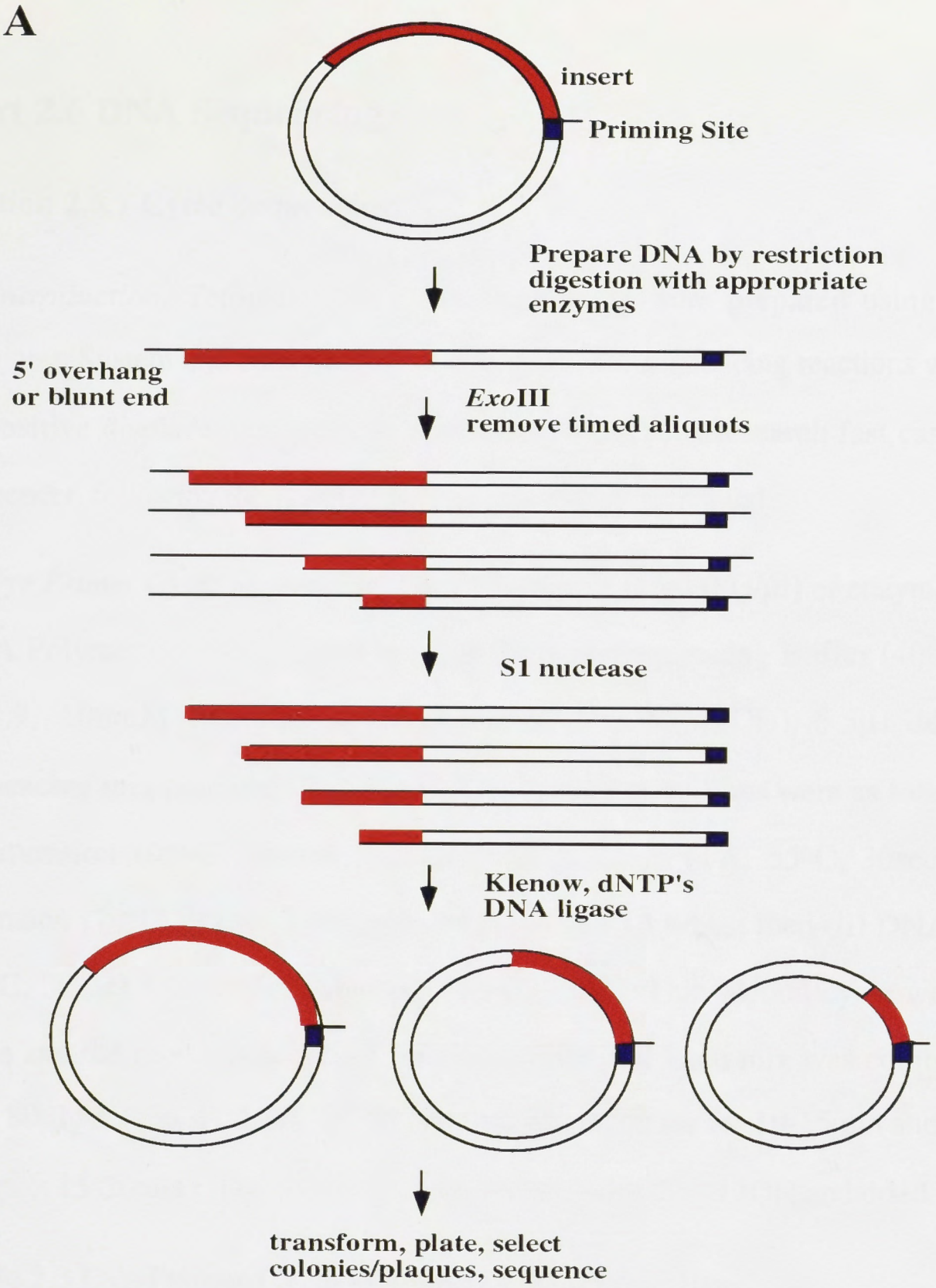
DNA mix was then warmed to 37°C (225bp/sec at 37°C; Figure 2.1B) and 382U of *Exo*III (Promega) added. After a lag time of 20-30secs a series of 20 aliquot's were removed (2.5µl) at 30sec intervals. These were added to the S1 nuclease mix and pipetted up and down briefly to deactivate the *Exo*III. After all the samples were taken the tubes were then placed at room temperature and S1 stop buffer (0.3M Tris base & 0.05M EDTA) added after 30min. To determine the extent of digestion and the success of the procedure 2µl from each time point was removed and run on a 1% agarose gel. Meanwhile 1µl of Klenow mix (30µl Klenow buffer & 3-5U Klenow) was added to the remainder of each sample, incubated (37°C, 3min) and 1µl of dNTP mix (0.125mM each of dATP, dCTP, dGTP & dTTP) added. Each tube was then left for a further 5min at 37°C, 40µl of Ligation mix (25 time points: 790µl ddH_2O, 100µl 10xLigase buffer, 100µL 50% PEG, 10µl 100mM DTT & 5U T4 DNA Ligase) added and each tube left at room temperature for 60min. 10µl of each sample was transformed into chemically competent *E. coli* DH5α cells, whilst the remainder of the overnight ligation mix was stored at 4°C.

Figure 2.1 The Promega Erase a base® Protocol.

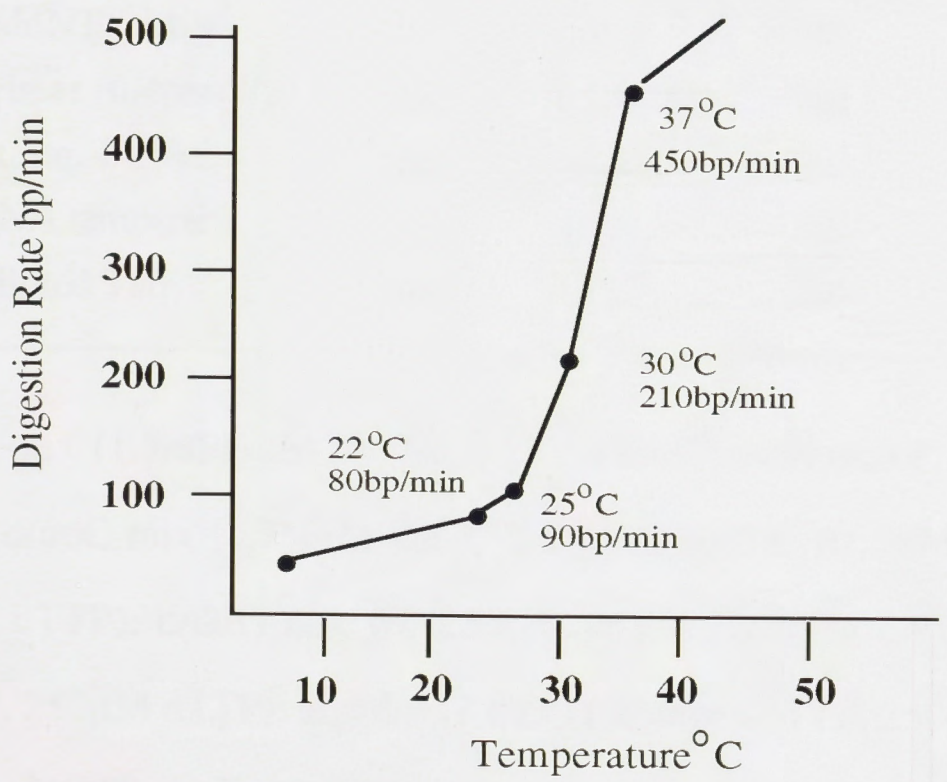
(A) Shown is a diagrammatic representation of the protocol used to generate timed deletion clones for sequencing. Initially the plasmid is cut with a specific endonuclease which linearises it and provides the site for primer protection. If the enzyme is a 3' cutter and therefore produces naturally endonuclease resistant ends, then a second enzyme is used to deprotect one end. However, if the enzyme produces 5' ends then both ends are first protected, before the second enzyme digest. In either case a series of timed deletions are taken using *Exo*III from the deprotected end and S1 nuclease used to 'chew back' the ssDNA overhang. The ends are then blunt ended, the fragments religated, transformed into chemically competent *E. coli* and prepared for sequencing.

(B) A graph showing the activity (digestion rate) of the *Exo*III exonuclease against temperature (Promega Catalogue).

A


insert
Priming Site
Prepare DNA by restriction digestion with appropriate enzymes
5' overhang or blunt end
ExoIII remove timed aliquots
S1 nuclease
Klenow, dNTP's DNA ligase
transform, plate, select colonies/plaques, sequence

B


Digestion Rate bp/min
500
400
300
200
100
10
20
30
40
50
Temperature °C
37 °C
450bp/min
30 °C
210bp/min
22 °C
80bp/min
25 °C
90bp/min

Part 2.6 DNA Sequencing

Section 2.6.1 Cycle Sequencing.

a) Introduction. Templates for cycle sequencing were prepared using the Wizard® Miniprep System and concentrated to 250ng/µl. The sequencing reactions were performed in positive displacement pippette tips, using a Corbett Research fast capillary thermal sequencer, following the Applied Biosystems technical manual.

b) Dye Primer Cycle Sequencing. For each sample 0.5µl (8U/µl) of enzyme (AmpliTaq® DNA Polymerase) was diluted in 1.0µl 5xCycle Sequencing Buffer (400mM Tris-HCl pH8.9, 100mM Ammonium Sulphate & 25mM $MgCl_2$), 5.5µl ddH_2O and the sequencing mix prepared (Table 2.3). The cycling conditions were as follows: (i) DNA denaturation (95°C, 30sec), annealing temperature (TA; 55°C, 30sec), and primer extension (70°C, 1min). This cycle was repeated 15 times; then (ii) DNA denaturation (95°C, 30sec) and primer extension (70°C, 1min). This second cycle was repeated 15 times and the products prepared for electrophoresis. Each mix was combined in a tube with 80µl 95% EtOH, 1.5µl of 3M NaOAc, placed on ice for 10-15min and then spun (12 000rpm; 15-30min). The pellet was then washed with 70% EtOH and dried.

Table 2.3 Dye Primer Cycle Sequencing Reaction Mixes.

Reagent	A	C	G	T
d/ddNTP Mix*	1µl	1µl	2µl	2µl
Primer (0.4pmol/µL)	1µl	1µl	2µl	2µl
5xSeq. Buffer	1µl	1µl	2µl	2µl
DNA template	1µl	1µl	2µl	2µl
Diluted Taq	1µl	1µl	2µl	2µl

*d/ddA mix (1.5mM ddATP, 62.5µM dATP, 250µM dCTP, 375µM c^7dGTP, 250µM dTTP); d/ddC mix (0.75mM ddCTP, 250µM dATP, 62.5µM dCTP, 375µM c^7dGTP, 250µM dTTP); d/ddG mix (0.125mM ddGTP, 250µM dATP, 250µM dCTP, 94µM c^7dGTP, 250µM dTTP); and d/ddT mix (1.25mM ddTTP, 250µM dATP, 250µM dCTP, 375µM c^7dGTP, 62.5µM dTTP).

c) Dye Terminator Cycle Sequencing. 5.0µl of ssDNA (0.5µg) or dsDNA (1.0µg) was added to 9.5µl of the terminator mix (Applied Biosystems). The primer was then added (0.8pmol for ssDNA or 3.2pmol for dsDNA) and the volume made up to 20µl with ddH_2O. The cycling conditions were as follows: DNA denaturation (95°C, 30sec), annealing temperature (TA; 50°C, 15sec), and primer extension (60°C, 4min). This cycle was repeated 25 times and the products prepared for electrophoresis. The dye terminators were extracted with 100µl phenol:H_2O:chloroform (68:18:14). The lower phase was discarded and the DNA precipitated with 15µl of 2M NaOAc pH4.5, 300µl of 100% EtOH and centrifugated. The pellet was then washed with 70% EtOH and dried.

Special Acknowledgments. The prepared sequencing products of TAQ dye primer and dye terminator cycle sequencing were given to the Biomolecular Resource Facility, ANU which ran the automated sequencer.

Section 2.6.2 Preparation of Single Stranded Template DNA.

Cultures of recombinant M13 phage were grown (6-8 hrs, 37°C) and precipitated from the solution with the addition of 4% PEG 6000/5M NaCl. After 5min centrifugation, DNA from the phage pellet was purified with TE-saturated:phenol:chloroform, precipitated and resuspended in 15µl TE (Sambrook *et al.*, 1989).

Section 2.6.3 Manual Sequencing.

a) ssDNA Sequencing. Sequencing reactions were carried out using the dideoxy chain termination procedure of Sanger *et al.*, 1977, but was modified to incorporate the use of T7 Polymerase (Pharmacia) instead of Klenow (Tabor & Richardson, 1987). To 5µl of template DNA was added 1µL of Universal primer and 1µl of 10x Annealing Buffer (30mM $MgCl_2$, 1M Tris pH 7.5 & 5M NaCl). This solution was then boiled and left to cool to room temperature. 1.5µl of the labelling mix, diluted 1/5, 0.5µl of α^{33}P-dATP (1 000Ci/mmol) and 1µl of T7 polymerase (3U/µl) was added to the annealing solution and incubated (5min, room temperature). 2.25µl was then aliquoted into prewarmed 1.25µl of the appropriate termination mix. After incubation (5min, 37°C) stop buffer was added.

b) dsDNA Sequencing. The dsDNA (2-4µg/µl) was first diluted in 18µl of ddH_2O and denatured by adding 2µl of 2M NaOH. The sample was then incubated for 5min at room temperature and 8µl of 5M NH_4OAc pH7.5, 100µl of 100% EtOH was added to recover the ssDNA. The supernatant was then removed, the pellet washed with 70% EtOH, dried *in vacuo* and dissolved in 7µl ddH_2O. 2µl of sequencing buffer (Pharmacia) and 1µl of primer (0.5pmol) was added and the solution heated (37°C, 20min). The following labelling and termination reactions were run exactly as described for ssDNA.

c) Denaturing Polyacrylamide Gel Electrophoresis (DNA Sequencing). The products from DNA sequencing reactions were separated on 6% denaturing polyacrylamide gels (400x200x0.4mm). Prior to loading the samples were heated (90°C, 3min), in order to prevent the occurrence of secondary structures which will disrupt the electrophoretic mobility of the DNA fragments. A solution containing 6% acrylamide (Acrylamide: Bisacrylamide 19:1, Sequagel, National Diagnostics), 8.3M urea, 90mM Tris-HCl pH8.2, 90mM Borate and 1mM EDTA was polymerised by adding 0.06% ammonium persulphate (APS; from a 10% stock solution) and 0.03% TEMED (N,N,N',N'-tetramethylethyldiamine) and poured between two prepared glass plates. If a limited amount of sequence data was required then the gel was run until the bromophenol blue dye present in the stop buffer reached the bottom of the gel. For longer stretches of information, or to provide a broad range of sequencing data, another run of the sample was loaded and the first run left until the xylene cyanol left the gel. The gel was fixed by applying a mixture of 20% EtOH, 10% acetic acid for 15min, transferred to 3MM Whatman paper, covered and dried at 80°C for 1hr, before being exposed to X-ray film.

CHAPTER 3 Targeting $G_{z\alpha}$ Expression *In Vivo*.

Part 3.1 Introduction.

Section 3.1.1 Gene Targeting.

Since the discovery that mammalian somatic cells possess the enzymatic machinery, and therefore the ability to mediate the introduction of non-replicating DNA molecules into the genome (Folger *et al.*, 1982; Lin *et al.*, 1984; Wake *et al.*, 1985), the study of such processes has lead to specific inactivation of a wide variety of genes *in vivo* (Gene Targeting; Appendix 3.1; Thomas *et al.*, 1986; Capecchi, 1989; Askew *et al.*, 1993; Barinaga, 1994; Shastry, 1994). Targeting gene expression has lead to both the creation of animal models for human genetic disorders [e.g. Gauchers's disease models (Tybulewicz *et al.*, 1992), Lesch-Nyhan syndrome (Kuehn *et al.*, 1987), and Cystic Fibrosis, (Dorin & Porteous, 1991)] and to a greater understanding of the role that some proteins play *in vivo* (Smithies *et al.*, 1985; Thomas *et al.*, 1986; Thomas & Capecchi, 1986;1987). Many gene targeting approaches begin with the inactivation of a single allele of the targeted gene in embryonic stem (ES) cells through the introduction of exogenous DNA (a linearised plasmid or retroviral vector) containing a copy of the same gene, interrupted by a marker or foreign genetic element (Robertson *et al.*, 1986; Thomas & Capecchi, 1987). Once this 'targeting construct' enters the genome of the ES cells *in vitro*, the non-functional copy of the gene which it carries recombines, and replaces the functional gene region (Riele *et al.*, 1992). Adaptations of this theme include: the hit-and-run approach or in-out technique (Hasty *et al.*, 1991; Shastry, 1994); the tag and exchange strategy (Askew *et al.*, 1993) and the Cre-lox system (Barinaga, 1994). ES cells are pluripotent *in vivo* and *in vitro* (Evans & Kaufman, 1981; Martin, 1981), a property which allows the researcher to inactivate the gene *in vitro*, and reintroduce the modified ES cells into mouse blastocysts (Robertson, 1991; Wood *et al.*, 1993a; 1993b), which grow within pseudopregnant female foster mothers and contribute to the formation of chimæric animals (mice possessing cells from both the native and foreign ES cell populations; Figure 3.1). Thus any modification made to the genome of the ES cells which is carried by the 'germline' chimæra (Gossler *et al.*, 1986; Robertson *et al.*, 1986) will be passed on to subsequent murine generations (Bradley *et al.*, 1984).

Figure 3.1 Gene Targeting.

Shown is a diagrammatic representation of a general approach taken used to disrupt the expression of genes *in vivo* (Courtesy of Dr Klaus Matthaei; Capecchi, 1989). A linearised targeting construct is introduced by electroporation into ES cells and allowed to recombine with the genome. Clones which possessing a stably and specifically deleted copy of the gene (+/-) are then isolated using selection media and confirmed with PCR. To produce a chimæric mouse, the C57BL/6 (black) ES cells are injected into the blastocyst from a white mouse strain (e.g. BALB/c). This combined (chimæric) blastocyst is then implanted into the uterus of a pseudopregnant female foster mother, where it is allowed to develop. The resultant progeny will contain cells which have originated from both the introduced C57BL/6 ES cells and the BALB/c ES cells native to the recipient blastocyst. The aim of producing a chimæric mouse is to obtain cells which are both heterozygous for the gene targeted and destined to contribute to the germline, so that the desired mutation can be passed on to succeeding generations. Whether or not this occurs is largely dependent upon the quality of the introduced ES cells and the technical expertise of the researcher. The only sure method of confirming the presence of germline chimæric animals is to breed them back into either the initial mouse strain, from which the introduced ES cells were obtained (e.g. C57BL/6) or another and to then screen these for the presence of progeny which are heterozygous (+/-) for the null mutation. These heterozygous mice can then be interbred to obtain: homozygous positive (+/+, 25%), heterozygous negative (-/+, 50%) and homozygous negative (-/-, 25%) progeny. If the mutation results in a lethal phenotype, then the resultant progeny will be either heterozygous negative (+/-, 33%) or homozygous positive (+/+, 66%; Thomas *et al.*, 1986; Capecchi, 1989; Robertson, 1991; Wood *et al.*, 1993a).

Blastocyst
C57Bl/6

Culture ES cells
in vitro

Targeting construct

neo^r

Electroporate
with targeting construct

Select for integration
and expand cells

Recipient Blastocyst
BalB/c

Implant
into foster mother

Chimaera

x

C57Bl/6

Chimaeric offspring

x

(+/-) (+/-)

(+/+)

(+/-) (+/-)

(-/-)

(+/+)

Homozygous Positive

(+/-)

neo^r

Heterozygous Negative

neo^r

(-/-)

neo^r

Homozygous Negative

Section 3.1.2 ES Cell Selection.

To screen for the presence of the targeting construct *in vitro,* the disrupting portion of DNA usually contains a selectable marker. The most common markers used confer resistance to the antibiotic neomycin (Neo^r; Gossler *et al.*, 1986; Robertson *et al.*, 1986). DNA introduced into ES cells has, however, a greater propensity for random integration rather than homologous or specific recombination (Figure 3.2; Roth & Wilson, 1985; Roth *et al.*, 1985). During homologous recombination the targeting construct enters the genome of the ES cell specifically and stably, in a process that coincides with the loss of any flanking regions, and the replacement of the native or functional gene with the modified/non-functional copy (Figure 3.2A; Capecchi, 1989). If, however, the targeting construct enters the genome randomly, and does not disrupt the targeted gene, then the flanking regions will have remained. To eliminate those clones which contain randomly inserted exogenous copies of the inactivated gene, a copy of the thymidine kinase (TK) gene (pgkTK or HSV-TK), is placed at the periphery of the targeting construct (Figure 3.2B). This confers sensitivity to gancyclovir (GANC), thus any cells containing a randomly inserted copy of the targeting construct will die when grown in media containing gancyclovir. Furthermore, because the TK gene will be lost following homologous recombination, those ES cells which contain a specifically integrated non-functional copy of the targeted gene should remain resistant to gancyclovir. Similarly since they also contain the pgkNeo gene, resistant to neomycin (G418; Folger *et al.*, 1984; Lin *et al.*, 1984; Smith & Berg, 1984; Smithies, 1984). This is, however, not always true (see below).

Section 3.1.3 PCR: a Sensitive Method of Eliminating False Positives.

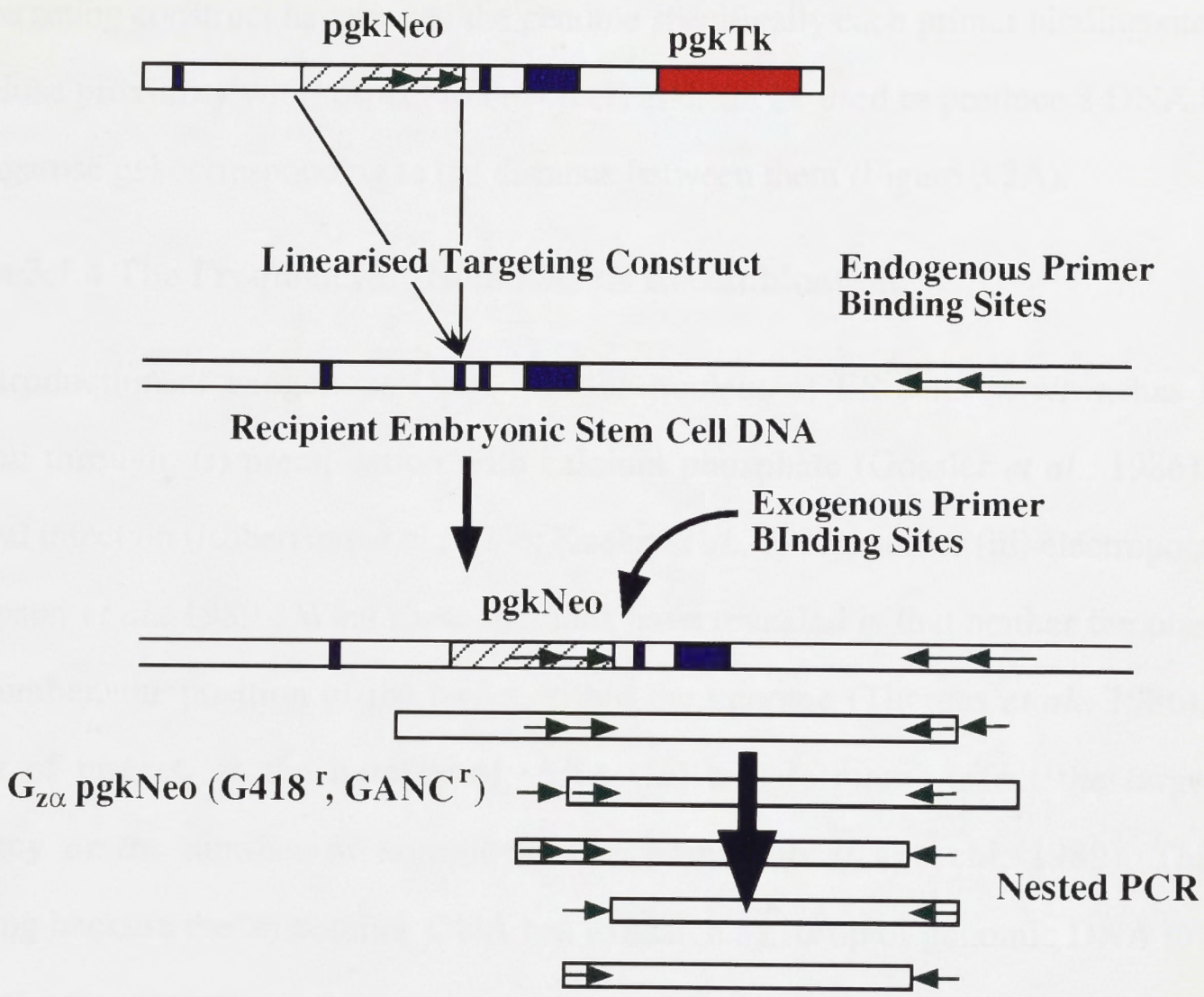
The use of antibiotic selection greatly enriches the number of clones which possess copies of the disrupted gene, however, a large number of these surviving ES cells still possess randomly incorporated copies of the targeting construct. They survive extinction because the TK gene has been inactivated through random mutation, or damage, during entry into the genome. A PCR (Mullis & Faloona, 1987) protocol which uses strategically placed priming sites close to the target and within the selectable marker, can provide a rapid and

Figure 3.2 Homologous Recombination vs Random Integration.

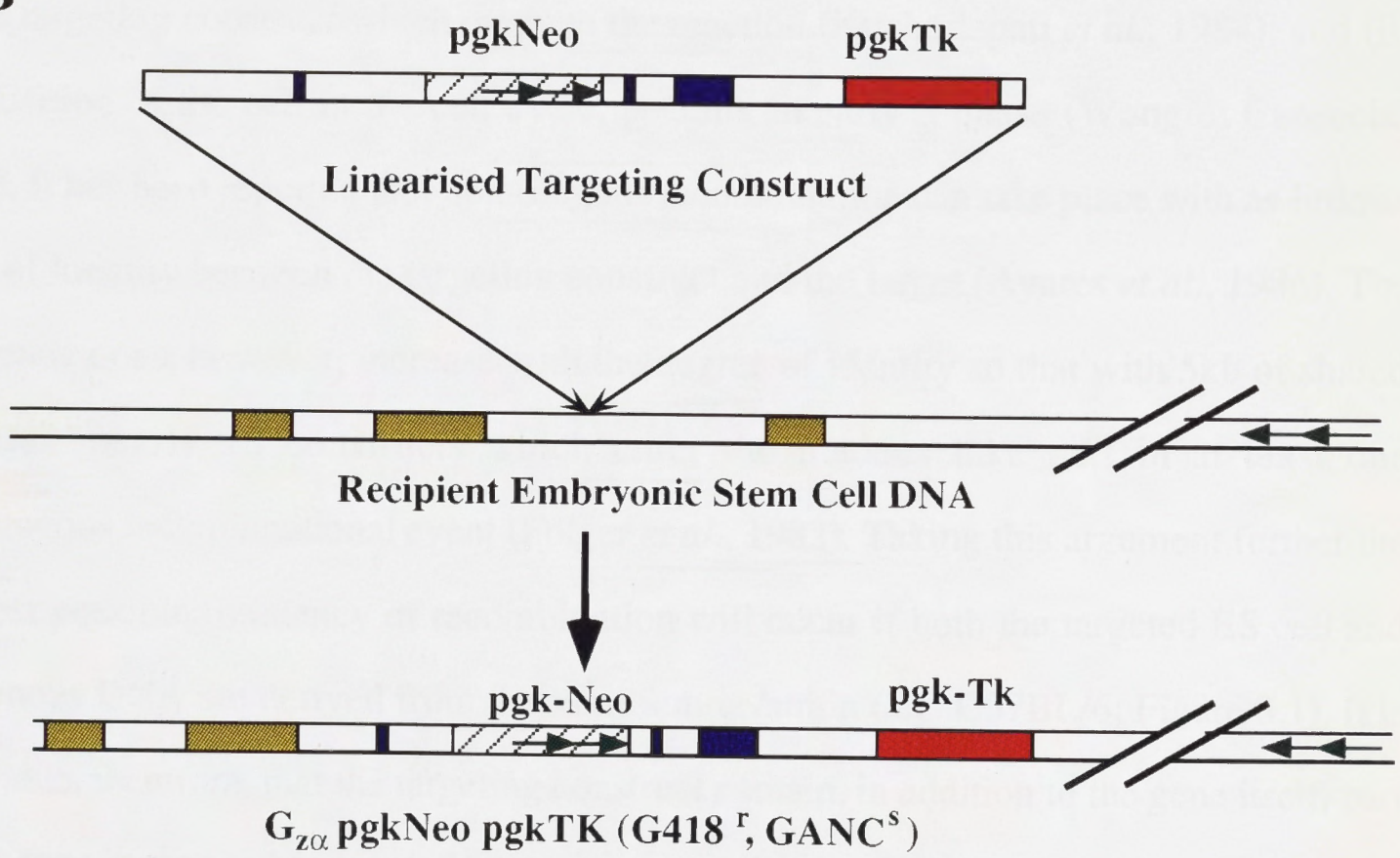
(A) During the process of homologous recombination a modified copy of the gene (targeting construct) enters the genome specifically carrying with it a selectable marker or disrupting element (e.g. pgkNeo). When this occurs the non-homologous ends are lost as are any markers placed at the periphery (e.g. pgkTK). Recombinant cells which possess a specifically integrated targeting construct can be easily isolated because of their resistance to both neomycin and gancyclovir ($G418^r$, $GANC^r$). Furthermore, both the foreign primer binding sites carried by the targeting construct, and those endogenous to the gene region targeted, can also be used to screen for specific integration.

(B) When random integration occurs the entire targeting construct (including the 'flanking' ends) enters the genome stably but non-specifically. So although these 'non-targeted' clones will be resistant to neomycin ($G418^r$) they will die in the presence of gancyclovir ($GANC^s$). If however, the pgkTK gene is damaged during entry these ES cell clones will be resistant to both gancyclovir and neomycin ($GANC^r$, $G418^r$), whilst the endogenous priming sites used to screen for homologous recombination (see above) will be absent (Thomas & Capecchi, 1986; 1987; Capecchi, 1989).

A


pgkNeo
pgkTk
Linearised Targeting Construct
Endogenous Primer
Binding Sites
Recipient Embryonic Stem Cell DNA
Exogenous Primer
BInding Sites
pgkNeo
Gzα pgkNeo (G418 r, GANC r)
Nested PCR

B


pgkNeo
pgkTk
Linearised Targeting Construct
Recipient Embryonic Stem Cell DNA
pgk-Neo
pgk-Tk
Gzα pgkNeo pgkTK (G418 r, GANC s)

efficient means of identifying and eliminating these false positives (Nitschke *et al.*, 1993). If the targeting construct has entered the genome specifically each primer binding site will lie in close proximity with respect to the other, and can be used to produce a DNA band on an agarose gel corresponding to the distance between them (Figure 3.2A).

Section 3.1.4 The Frequency of Homologous Recombination.

The introduction of exogenous DNA into the nucleus of ES cells *in vitro*, has been achieved through: (i) precipitation with calcium phosphate (Gossler *et al.*, 1986); (ii) retroviral infection (Robertson *et al.*, 1986; Kuehn *et al.*, 1987); and/or (iii) electroporation (Thompson *et al.*, 1989). What these methods have revealed is that neither the plasmid copy number, the position of the target within the genome (Thomas *et al.*, 1986), the number of targets, or the number of successful transformants affect the targeting frequency or the number of homologous recombinants (Capecchi, 1989). This is surprising because the 'in coming' DNA has to search $3x10^9$bp of genomic DNA to find the target gene, however, it does mean that these experiments can be done with very few molecules per cell (Capecchi, 1989). The rate/frequency of homologous recombination is in fact dependent upon: (i) the degree of sequence identity between the target and the ends of the targeting construct which mediate the reaction (Kucherlapati *et al.*, 1984); and (ii) the position of the cell in the cell cycle, peaking in early S phase (Wong & Capecchi, 1987). It has been reported that homologous recombination can take place with as little as 25bp of identity between the targeting construct and the target (Ayares *et al.*, 1986). The frequency does, however, increase with the degree of identity so that with 5kb of shared sequence nearly all constructs which enter the nucleus take part in at least one homologous recombinational event (Folger *et al.*, 1982). Taking this argument further the greatest possible frequency of recombination will occur if both the targeted ES cell and exogenous DNA are derived from the same source/strain (e.g. C57BL/6; Figure 3.1). It is preferable, therefore, that the targeting construct contain, in addition to the gene itself, part of the gene region to be targeted (e.g. an exon and part of the surrounding genome, Part 3.2, p65).

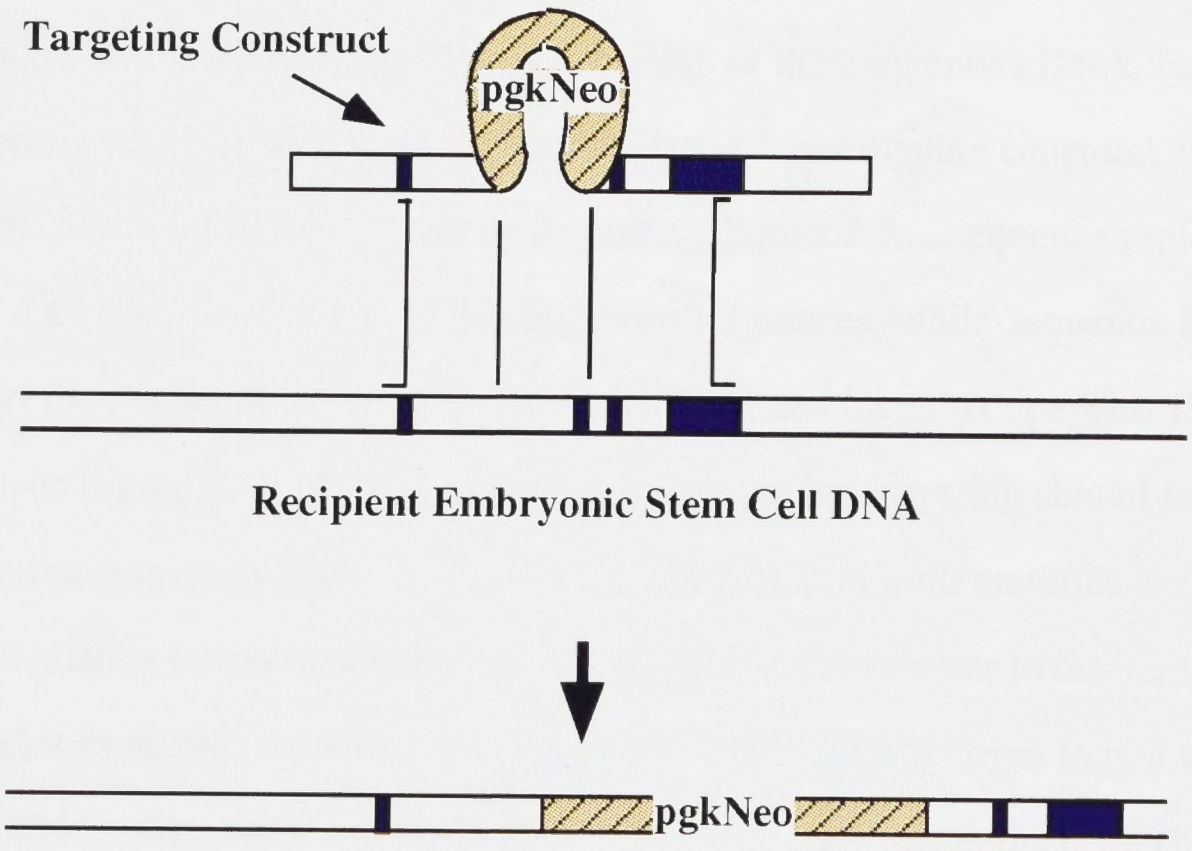
Figure 3.3 Replacement vs Insertion Targeting.

Both 'replacement' and 'insertion' targeting events are largely dependent upon and describe the construction of the 'targeting' construct.

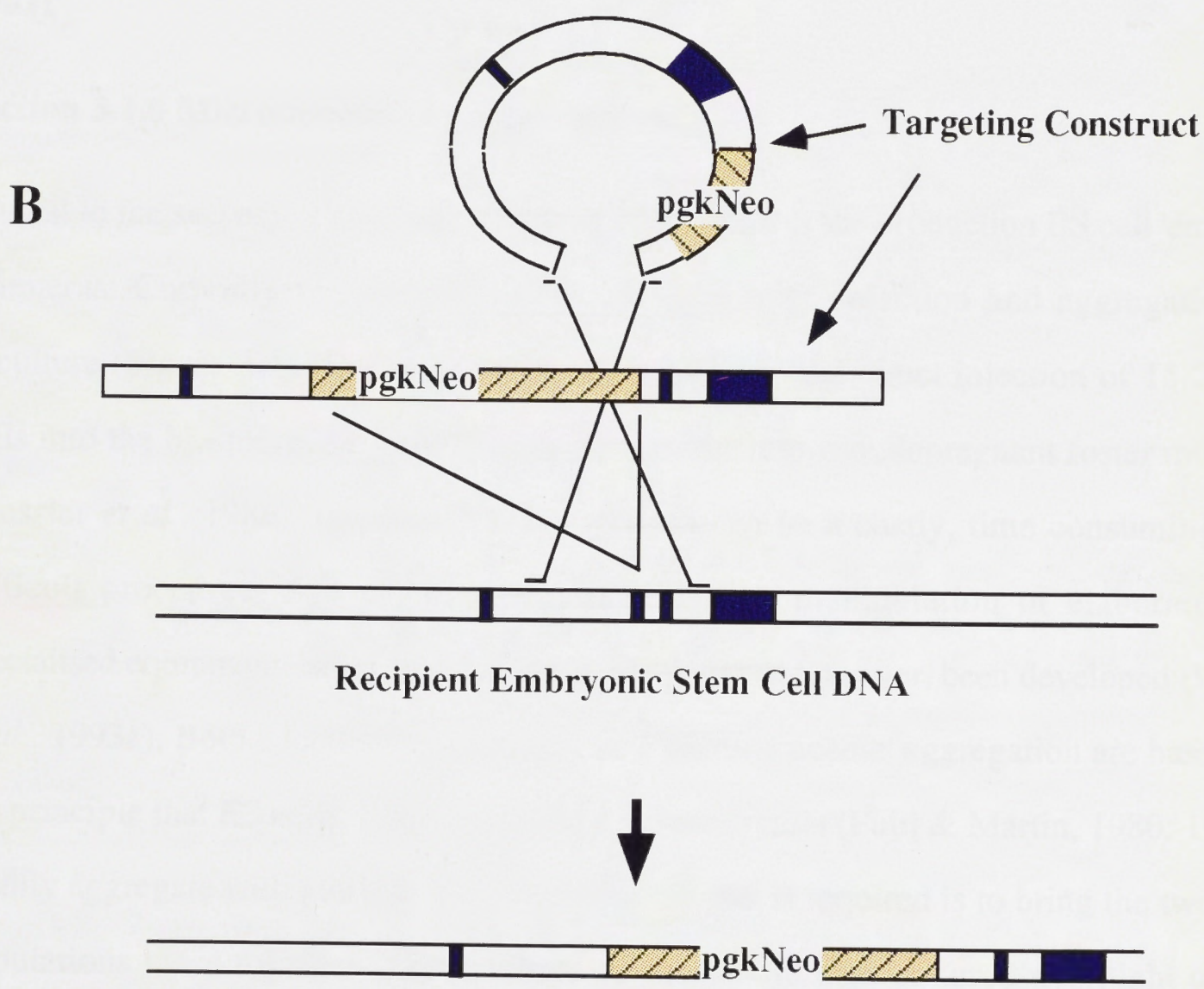
(A) During 'replacement' targeting the selection marker replaces an excised portion of the cloned gene. This allows the homologous regions of the introduced construct to come alongside and line up with the corresponding genomic sequence.

(B) During 'insertion' targeting, often a single restriction site is chosen and used to introduce the selection marker. This means that during homologous recombination the marker needs to loop away from the site of recombination in order to allow the homologous ends of the targeting construct to pair with the targeted gene region (Capecchi, 1989).

A


Targeting Construct
pgkNeo
Recipient Embryonic Stem Cell DNA
pgkNeo

B


Targeting Construct
pgkNeo
pgkNeo
Recipient Embryonic Stem Cell DNA
pgkNeo

Section 3.1.5 Sequence Insertion vs Sequence Replacement Targeting.

Both sequence insertion targeting and deletion or replacement targeting describe the recombinational event which follows transformation of the exogenous DNA. Each are a form of targeting which depend upon the construction of the targeting construct itself, and the placement of the selectable marker or disrupting element: ".....sequence replacement vectors replace endogenous DNA with exogenous sequences, while sequence insertion vectors insert the exogenous sequence into the endogenous locus" (Capecchi, 1989). At first glance (see Figure 3.3), it would appear that replacement targeting should result in a greater targeting frequency, however, it has been shown that both methods are equally efficient at mediating homologous recombinational events and appear to respond equally well to changes in identity between the exogenous DNA and the target (e.g. a two fold increase in (%) identity results in a 20-fold increase in the targeting frequency; Thomas & Capecchi, 1987; Capecchi, 1989). Because of this, the choice of targeting vector is largely determined by the restriction sites available (Thomas & Capecchi, 1987; Abeliovich *et al.*, 1993).

Section 3.1.6 Microinjection vs Aggregation.

Crucial to the success of any gene targeting experiment is the production ES cell 'embryo' chimæras. Currently two methods exist, through microinjection and aggregation or coculture (Figure 3.4). The most successful method is the direct injection of 15-20 ES cells into the blastocoel cavity followed by transfer into pseudopregnant foster mothers (Gossler *et al.*, 1986). In practice this approach can be a costly, time consuming and difficult procedure, that requires dexterity and the manipulation of elaborate and specialised equipment. Several simplified methods have, however, been developed (Wood *et al.*, 1993a). Both coculture aggregation and 'darning needle' aggregation are based on the principle that ES cells, like embryonic carcinoma cells (Fujii & Martin, 1980; 1983), readily aggregate with morulae and, therefore, all that is required is to bring the two cell populations close together. The resultant chimæras are then cultured overnight to the blastocyst stage before they are implanted into pseudopregnant female recipients. A comparison of the two methods (aggregation vs injection) shows that there is, however, no

real difference in the number of germline chimæras produced (Table 3.1; Wood *et al.*, 1993a). This means that the method of choice is largely dependent upon the availability of money, equipment and technical expertise.

Table 3.1 Germline Chimæra Production by 'Darning Needle' Aggregation of Morulae vs Blastocyst Injection.

(Table taken from: Wood *et al.*, 1993a)

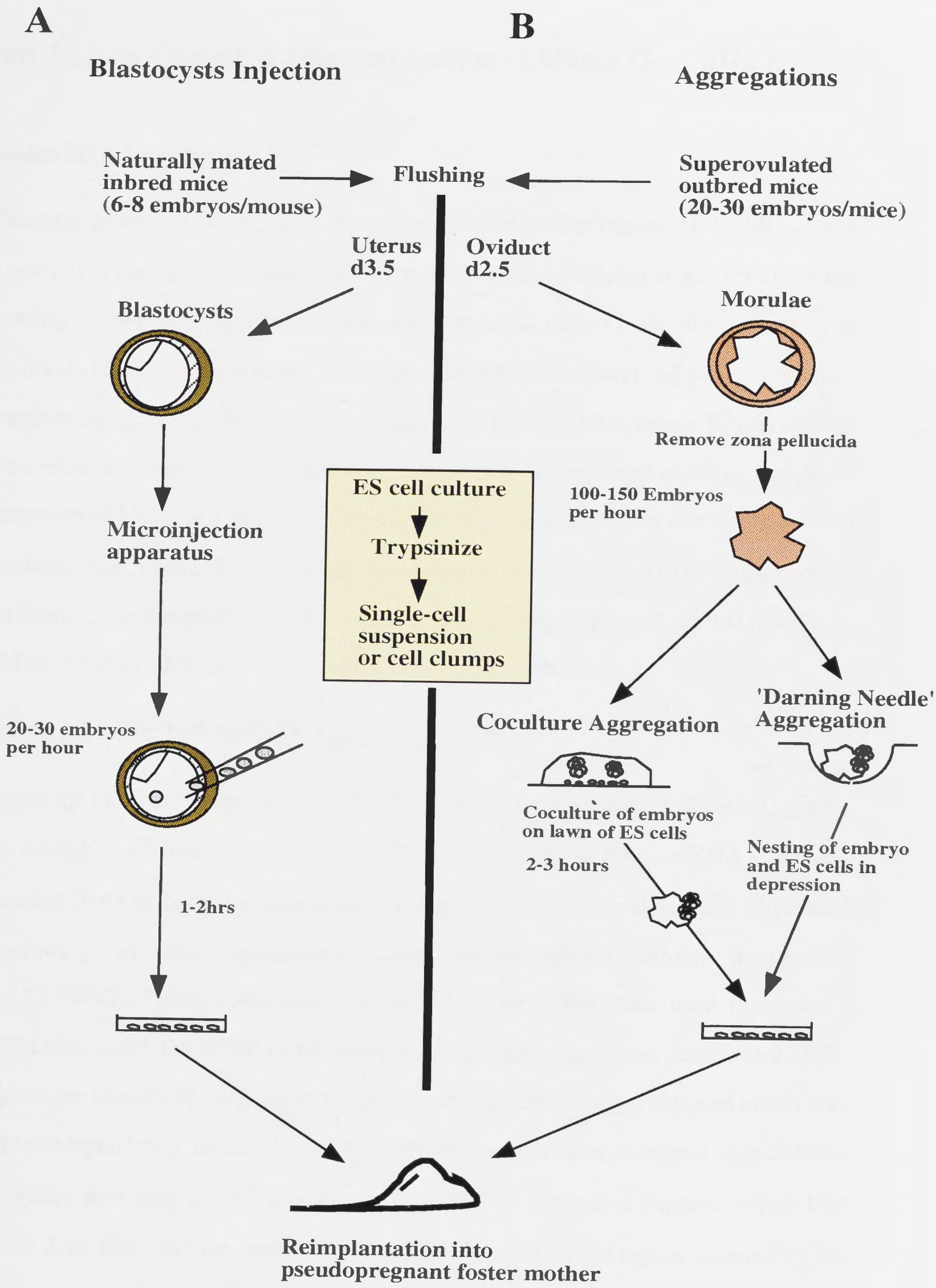
Technique	Embryos transferred	Newborns	Chimæras		Germline Transmitters
			Males	Females	
Morulae aggregation	321	89(28%)	31(9.6%)	1(0.3%)	11(3.4%)
Blastocyst injection	631	250(40%)	72(11.4%)	23(3.6%)	24(3.8%)

Figure 3.4 Coculture vs Direct Injection.

Shown in the figure is a diagrammatic representation of both coculture and direct injection techniques used to obtain chimæric animals from ES cells in culture (Wood *et al.*, 1993a).

(A) In the case of direct injection, the cells are forced into the blastocoel cavity through a specially made, drawn out glass pipette. This is technically difficult and problems often arise if the cavity is not properly penetrated, or if ES cells lyse increasing the viscosity of the media and causing material to 'stick' to the outside of the glass pipette. The 'chimæric' blastocyst is then allowed to incubate for 1-2hrs before being implanted into a pseudopregnant female foster mother.

(B) In the case of those techniques involving aggregation, morulae are stripped of their outer layer (zona pellucida), and allowed to coculture or aggregate with a lawn of the targeted ES cells, prepared earlier. The closer the contact, the greater the chance that the morulae will take up the free ES cells (e.g. 'Darning Needle' Aggregation). The cells are then cultured and the 'chimæric' morulae reimplanted into pseudopregnant female foster mothers (Wood *et al.*, 1993b).


A
Blastocysts Injection
B
Aggregations
Naturally mated
inbred mice
(6-8 embryos/mouse)
Flushing
Superovulated
outbred mice
(20-30 embryos/mice)
Uterus
d3.5
Oviduct
d2.5
Blastocysts
Morulae
Remove zona pellucida
ES cell culture
Trypsinize
Single-cell
suspension
or cell clumps
100-150 Embryos
per hour
Microinjection
apparatus
20-30 embryos
per hour
Coculture Aggregation
'Darning Needle'
Aggregation
Coculture of embryos
on lawn of ES cells
2-3 hours
Nesting of embryo
and ES cells in
depression
1-2hrs
Reimplantation into
pseudopregnant foster mother

Part 3.2 The Cloning & Characterisation of Mouse $G_{z\alpha}$ ($MG_{z\alpha}$).

Section 3.2.1 Introduction.

Whilst the genetic organisation of the coding and non-coding regions of the $MG_{z\alpha}$ gene is unknown, certain assumptions can be made, based upon what is known about the orthologous human ($HG_{z\alpha}$) and rat ($RG_{z\alpha}$) genes. $MG_{z\alpha}$ differs from other G_{α} genes, as it is divided into only three exons, a 5' untranslated region (exon1) and two exons which contain translated sequence (exon2 & exon3, Figure 3.5A). As this unique pattern appears to be relatively well conserved between species, the decision was made to 'knockout' expression of $MG_{z\alpha}$ at a point close to start of translation (ATG), by disrupting the first translated exon, exon2. The following sections detail the steps taken to completely clone and characterise that portion of the mouse genome surrounding exon2, so that it could be used to construct a $MG_{z\alpha}$, C57BL/6 specific targeting construct.

Section 3.2.2 The Isolation of $MG_{z\alpha}$ Genomic Clones.

Despite the fact that neither the complete sequence nor the organisation of the $MG_{z\alpha}$ gene was available, a partial complementary DNA (cDNA) of the $MG_{z\alpha}$ mRNA ($MG_{z\alpha}P$), spanning parts of both the second and third exons had been isolated and sequenced previously by this laboratory. This partial cDNA clone, designated pBSK(+)$MG_{z\alpha}$P(696bp,4BF/3R) (Figure 4.1; Leck, 1993) was used to screen a C57BL/6 mouse λ DASH®II phage genomic library (λM; Stratagene; Appendix 2.2). To confirm the identity of the λ phage mouse genomic clones ($\lambda MG_{z\alpha}$) obtained in this way and to independently isolate the exon2 specific $\lambda MG_{z\alpha}$ clones, a second round of low stringency screening (55°C) was employed, using the consensus oligonucleotide 1BF (Table 2.1). Note that the binding site for 1BF lies outside the region spanned by the $MG_{z\alpha}P$ 'probe' (Figure 3.5B; Leck, 1993).

Section 3.2.3 Restriction Mapping the Isolated $\lambda MG_{z\alpha}$ Clones.

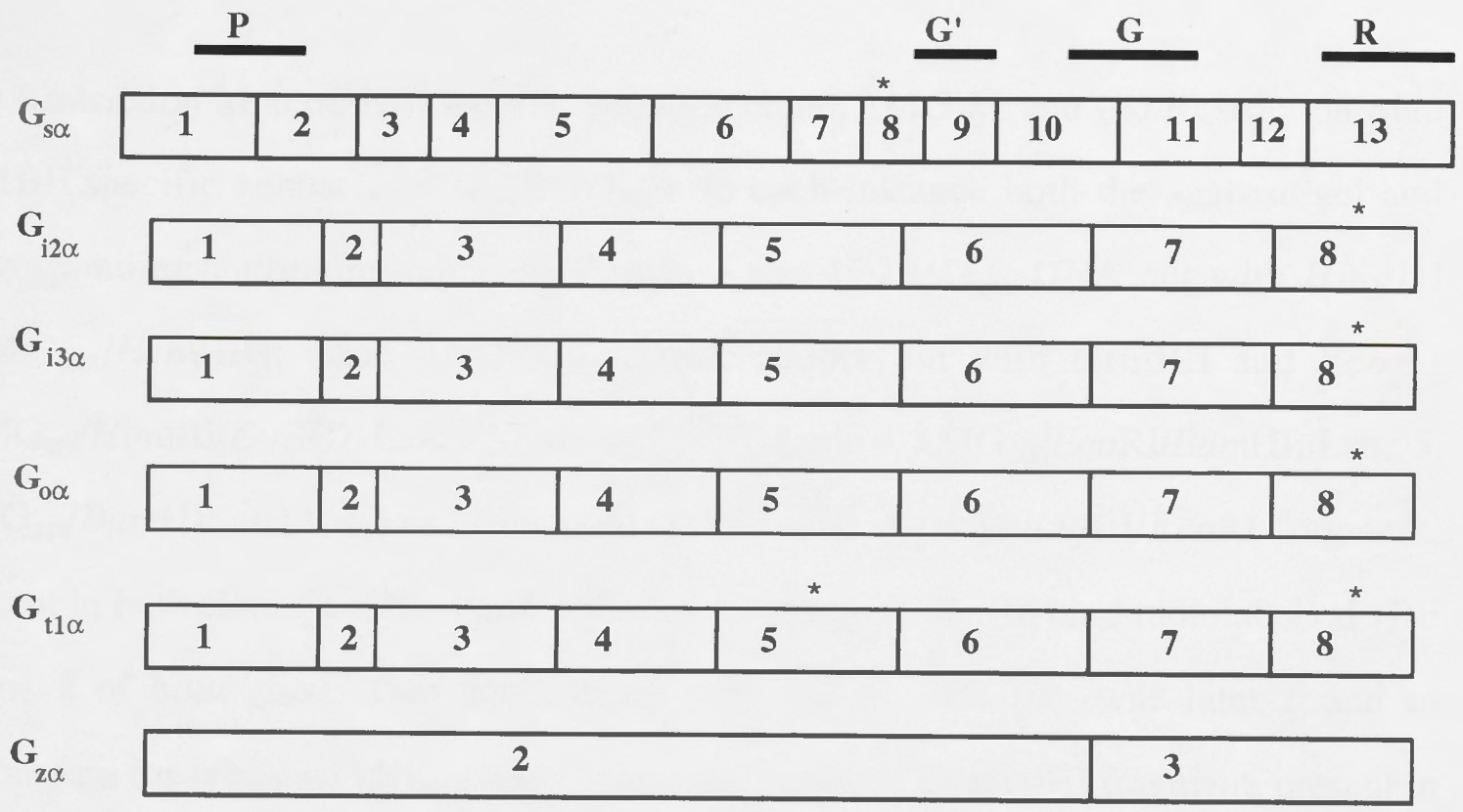
a) Mapping the Site of 1BF Hybridisation. To identify the position of $MG_{z\alpha}$ exon2 the DNA from each of the $\lambda MG_{z\alpha}$ clones, $\lambda MG_{z\alpha}1$, $\lambda MG_{z\alpha}2$, $\lambda MG_{z\alpha}4$ and $\lambda MG_{z\alpha}11$ was

Figure 3.5 The Organisation of the $G_{z\alpha}$ Gene.

(A) Shown in this figure are the known coding regions of some of the G_{α} genes. According to rat and human studies the $G_{z\alpha}$ gene possesses two coding exons (exon2 & exon3), and one 5' non-coding exon (exon1). This contrasts with many other G_{α}'s including $G_{i2\alpha}$, which although it shares a high degree of sequence similarity with $G_{z\alpha}$, is made up of seven coding and one 3' non-coding exon (Itoh *et al.*, 1988; Kozasa *et al.*, 1988; Fong *et al.*, 1988; Matsuoka *et al.*, 1990; Raport *et al.*, 1989). Pertussis and cholera toxin ADP-ribosylation sites are also indicated (*), as are the highly conserved regions P, G', G and R. The G and P regions are thought to involved in GTP binding and hydrolysis, respectively, whilst the region marked R is believed to be involved in receptor coupling (Matsuoka *et al.*, 1990).

(B) Both the $RG_{z\alpha}$ and $HG_{z\alpha}$ coding and non-coding exons are separated by large regions of the genome (>20kb, Fong *et al.*, 1988; Matsuoka *et al.*, 1990). The translated (or protein coding) regions of each exon are shaded. Also shown are the start sites of translation and transcription (arrows) as well as the approximate positions of each of the consensus oligonucleotides (1BF, 4BF & 3R). Both 4BF (exon2) and 3R (exon3) form the 5' and 3' boundaries of the partial $MG_{z\alpha}$ cDNA probe (Leck, 1993), whilst 1BF was used to identify the exon2 specific $\lambda MG_{z\alpha}$ clones, $\lambda MG_{z\alpha}1$, $\lambda MG_{z\alpha}2$, $\lambda MG_{z\alpha}4$ & $\lambda MG_{z\alpha}11$.

A


P
G'
G
R
$G_{s\alpha}$
1 2 3 4 5 6 7 8 9 10 11 12 13
$G_{i2\alpha}$
1 2 3 4 5 6 7 8
$G_{i3\alpha}$
1 2 3 4 5 6 7 8
$G_{o\alpha}$
1 2 3 4 5 6 7 8
$G_{t1\alpha}$
1 2 3 4 5 6 7 8
$G_{z\alpha}$
2 3
*

B

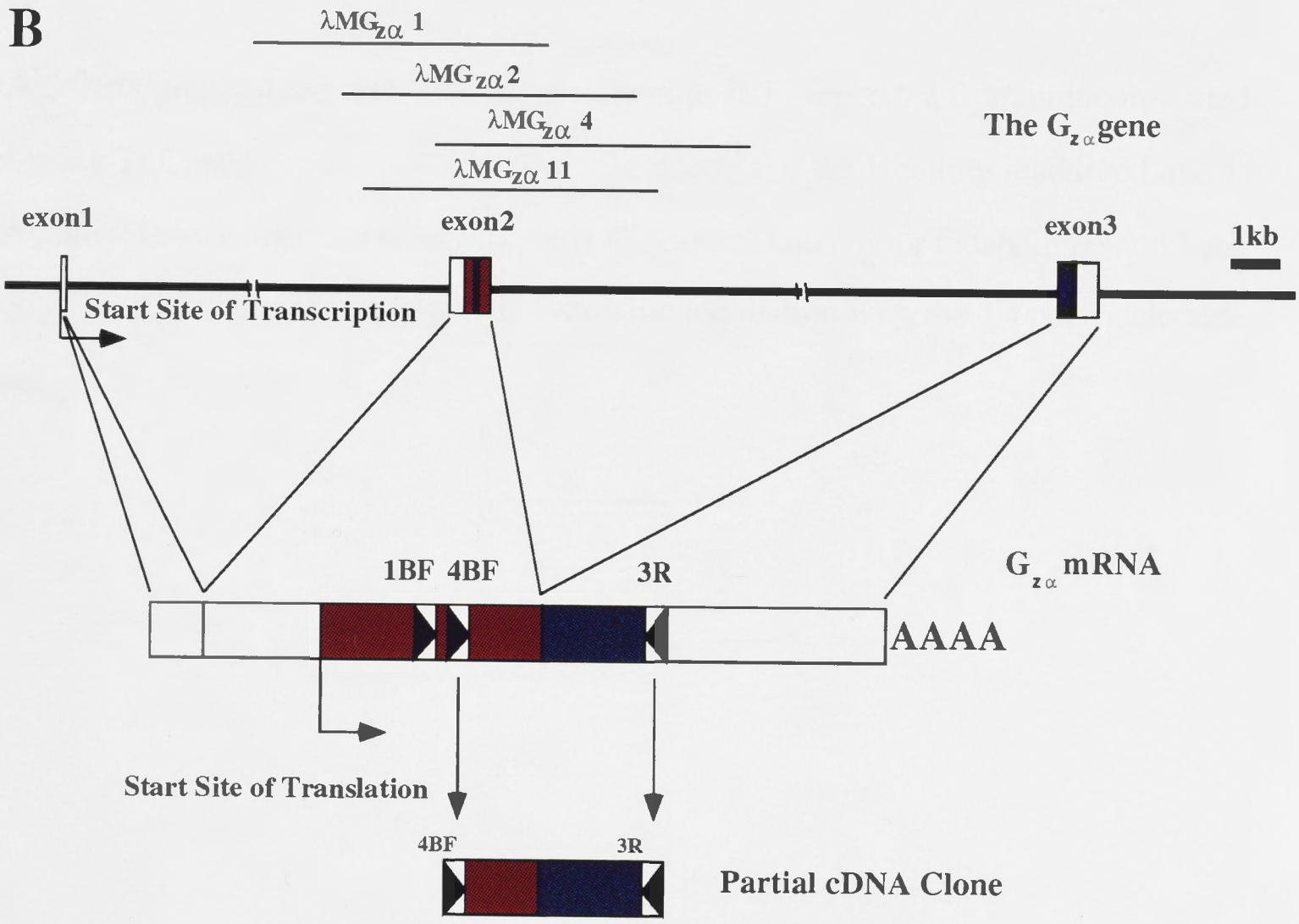
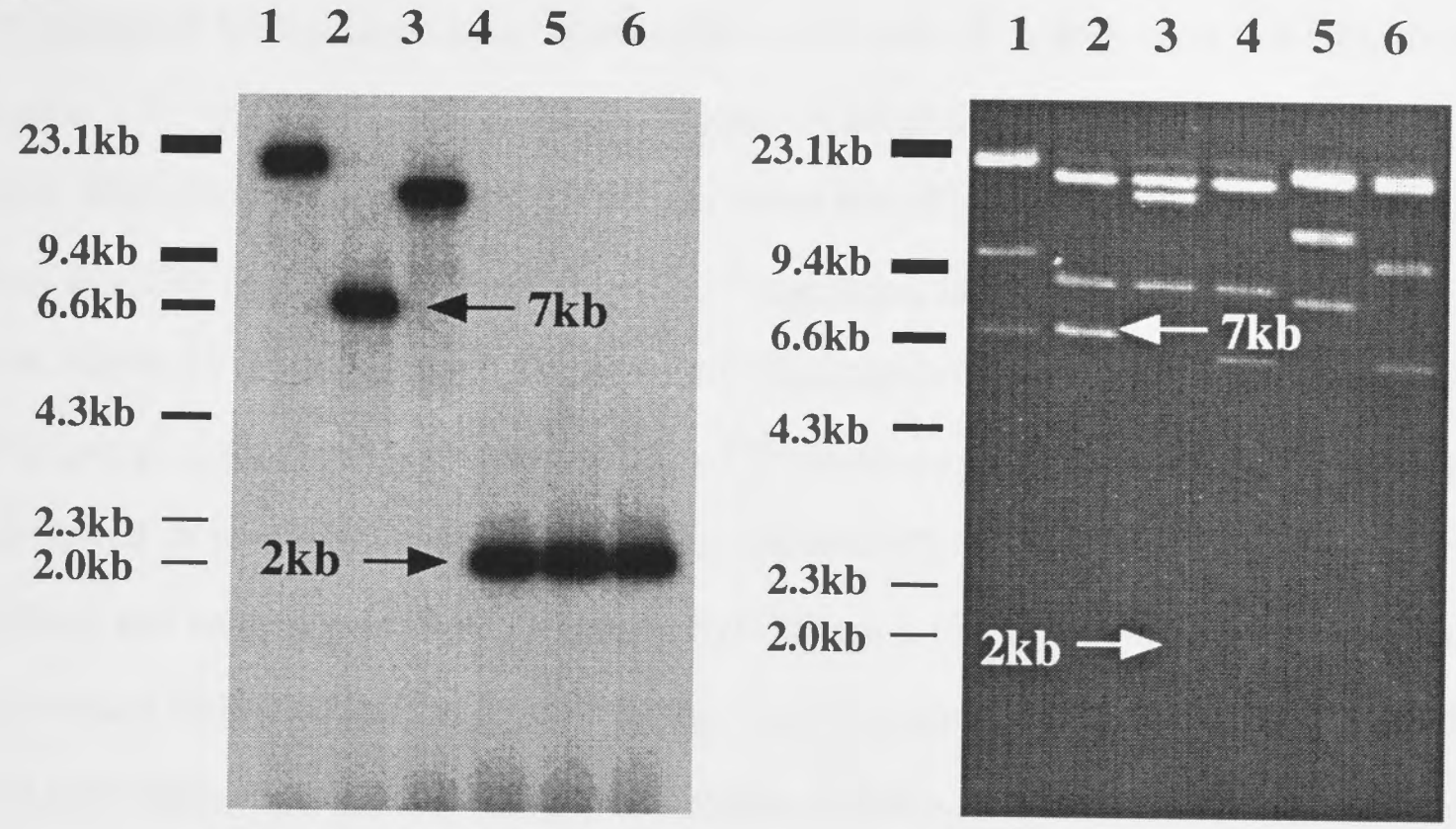

λMG$_{z\alpha}$ 1
λMG$_{z\alpha}$ 2
λMG$_{z\alpha}$ 4
λMG$_{z\alpha}$ 11
The $G_{z\alpha}$ gene
exon1
exon2
exon3
1kb
Start Site of Transcription
1BF 4BF
3R
$G_{z\alpha}$ mRNA
AAAA
Start Site of Translation
4BF
3R
Partial cDNA Clone

Figure 3.6 Mapping the 1BF Positive $\lambda MG_{z\alpha}$ Clones

(A) Restriction Map of 1BF specific genomic clone, $\lambda MG_{z\alpha}1$ and **(B)** Restriction Map of 1BF specific genomic clone, $\lambda MG_{z\alpha}4$. In each instance both the agarose gel and corresponding autoradiograph are shown. Lane 1, $\lambda MG_{z\alpha}$ DNA cut with *Hin*dIII ($\lambda MG_{z\alpha}$/*Hin*dIII); Lane 2, $\lambda MG_{z\alpha}$ DNA doubly cut with *Hin*dIII and *Eco*RI ($\lambda MG_{z\alpha}$/*Hin*dIII/*Eco*RI); Lane 3, $\lambda MG_{z\alpha}$/*Eco*RI; Lane 4, $\lambda MG_{z\alpha}$/*Eco*RI/*Bam*HI; Lane 5, $\lambda MG_{z\alpha}$/*Bam*HI; and Lane 6, $\lambda MG_{z\alpha}$/*Bam*HI/*Hin*dIII. A 7kb *Hin*dIII/*Eco*RI fragment, present in both clones $\lambda MG_{z\alpha}1$ and $\lambda MG_{z\alpha}4$, is clearly shown to bind radiolabelled 1BF (Lane 2 of both gels). This band, designated $MG_{z\alpha}2$(7.0H/E), was later found to encompass the whole of $MG_{z\alpha}$ exon2. Also shown is the 2kb *Bam*HI fragment, present in both clones, which although difficult to see following separation by agarose gel electrophoresis, is visible following hybridisation with 1BF. The larger bands (>15kb), are the arms of the λ phage cloning vector. The size marker used, λ/*Hin*dIII, is reproduced on the left.

(C) Autoradiograph of radiolabelled oligonucleotide 1BF, separated from unincorporated label using TLC, and conducted to ascertain the success of the labelling reaction. Lane 1, is the control lane, containing unincorporated nucleotide, taken prior to labelling; and Lane 2, contains 1μL of kinase reaction mix, following incubation with the T4 polynucleotide kinase.

A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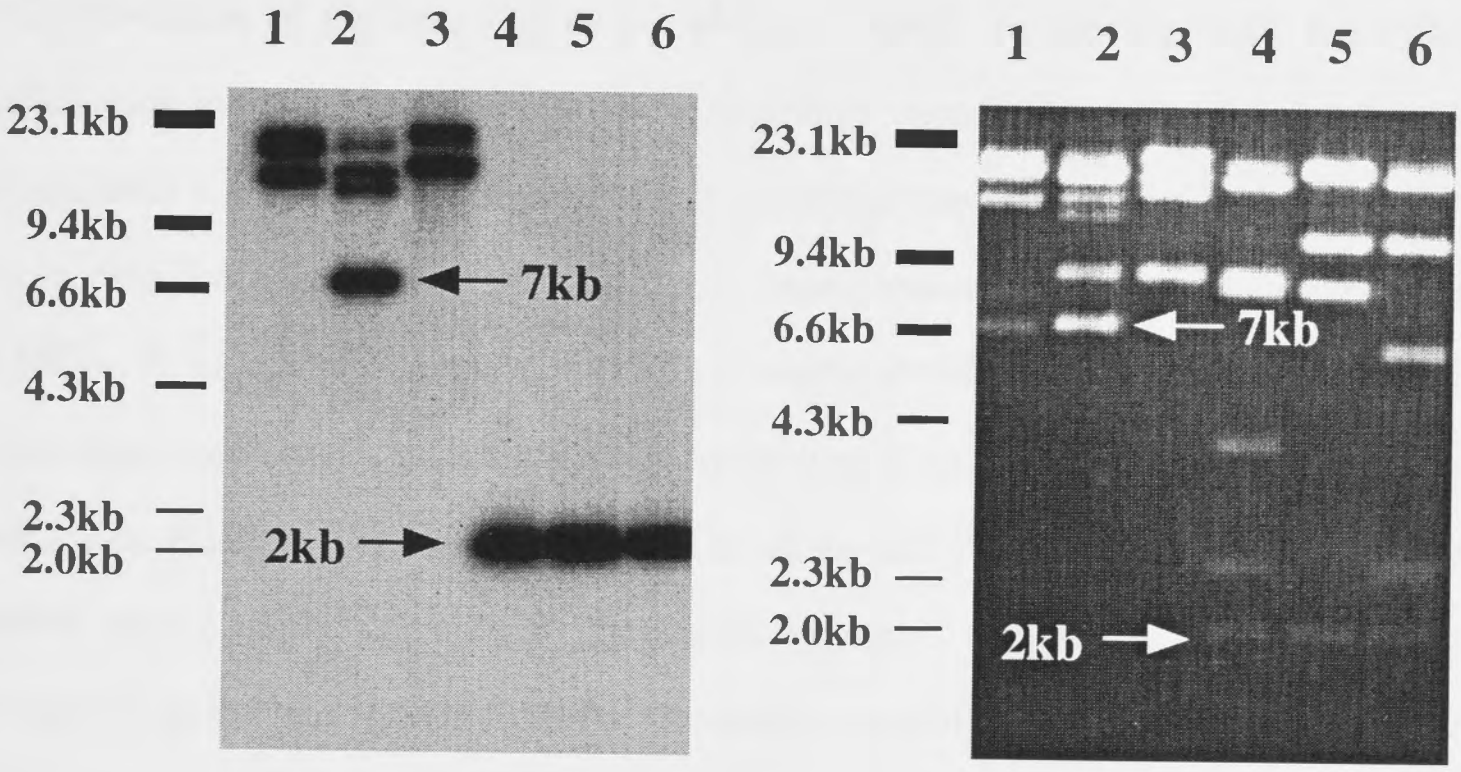
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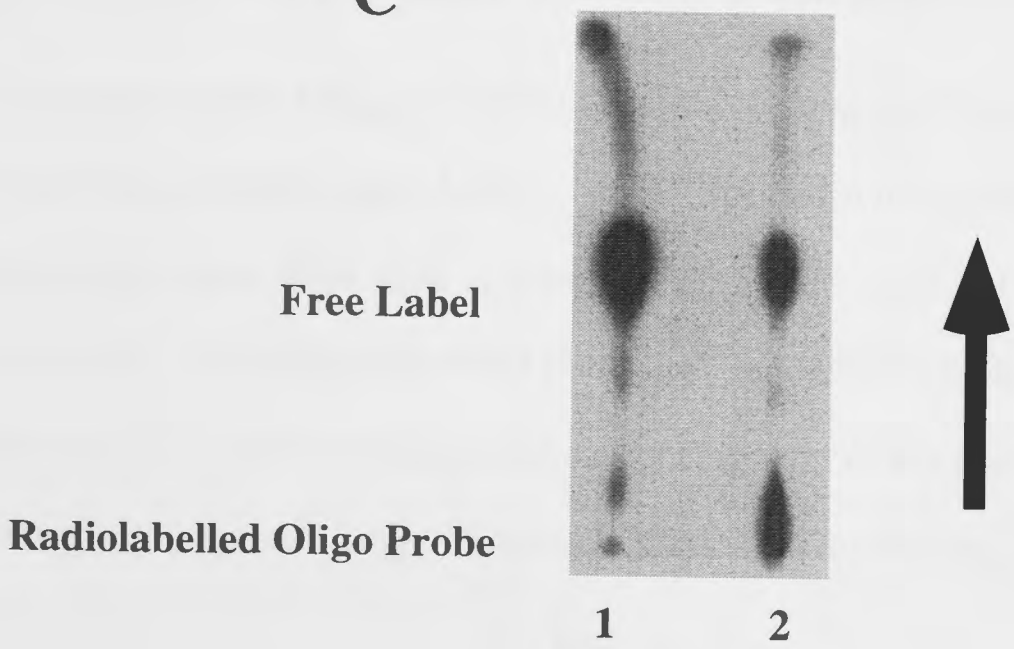
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2

first prepared from a large scale liquid culture (Section 2.2.2, p34), and digested (1μg; Section 2.3.1, p38) with the specific endonucleases *Hin*dIII, *Eco*RI, and *Bam*HI* (Section 2.1.2, p30), chosen because they do not cut either the $RG_{z\alpha}$ or $HG_{z\alpha}$ coding sequences. Thus any 1BF positive restriction fragment, which may later be isolated should contain most, if not all, of $MG_{z\alpha}$ exon2. The restriction fragments were then separated by agarose (1%) gel electrophoresis (Section 2.2.3, p35), photographed under UV illumination, transferred to positively charged nylon membrane (PCNM) using the alkaline transfer method, and subjected to high stringency hybridisation (63°C; Section 2.4.3, p45) with endlabelled 1BF (Section 2.4.1, p44). In this way a number of 1BF binding DNA bands were identified, including a 2kb *Bam*HI fragment, which is itself encompassed by a larger, 7kb region of the genome flanked by *Hin*dIII and *Eco*RI (7kbH/E) restriction sites (Figure 3.6; 3.10).

b) Confirmation of the exon2 Specific $\lambda MG_{z\alpha}$ Clones. Following high stringency hybridisation with radiolabelled 1BF the membranes were stripped (Section 2.4.3, p45), and reprobed (63°C) with endlabelled exon3 specific primer 3R (Figure 3.5B; Table 2.1). As expected, from the high degree of sequence and organisational conservation between the $MG_{z\alpha}$, $RG_{z\alpha}$ and $HG_{z\alpha}$ genes, none of the exon2 specific $\lambda MG_{z\alpha}$ clones were found to also contained exon3, which they may have done if exon3 lay in close proximity to exon2. The decision was, therefore, made to clone and characterise the 1BF positive, putative exon2 containing 7kbH/E genomic fragment, designated $MG_{z\alpha}2(7.0H/E)$, because it is both large enough to entirely contain a genomic copy of $MG_{z\alpha}$ exon2 (~1kb) and small enough to be easily prepared and sequenced.

Section 3.2.4 The Cloning & Characterisation of a 7kb $MG_{z\alpha}$ Subclone.

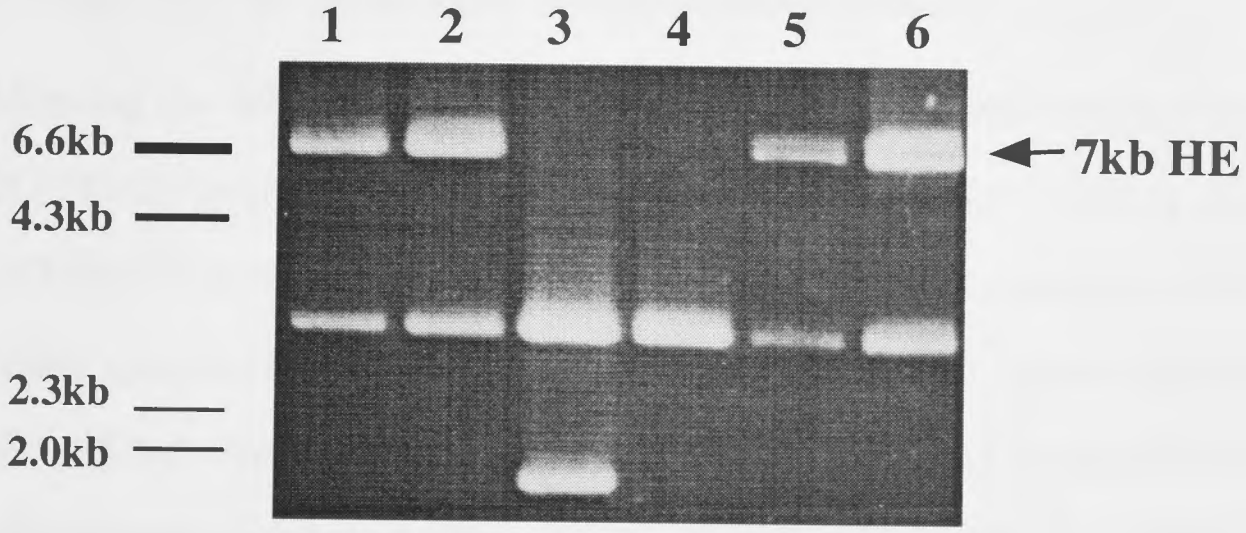
a) Isolation of the $MG_{z\alpha}2(7.0H/E)$ Genomic Fragment. The 1BF positive, putative exon2 containing genomic clone $\lambda MG_{z\alpha}1$, was digested with *Hin*dIII and *Eco*RI. The resulting DNA fragments were then cloned into *Hin*dIII/*Eco*RI cut phagemid vector, pBSK(+) (Appendix 2.1) and transformed (Section 2.3.7, p40) into electrocompetent *E.coli* DH5α (Section 2.1.1, p30). Clones possessing a fragment of the correct size, were then identified through colony cracking (Section 2.3.8, p41), restriction analysis (Figure 3.7A), and

Figure 3.7 Restriction Analysis of the $MG_{z\alpha}2$(7.0H/E) Clone.

(A) Shown are some of the plasmids obtained following 'shot gun' cloning of the *Hin*dIII/*Eco*RI cut λ$MG_{z\alpha}1$ digest. Lane 1, 17HE pBSK(+)$MG_{z\alpha}2$(7.0H/E); Lane 2, 6HE pBSK(+)$MG_{z\alpha}2$(7.0H/E); Lane 3, 11HE contains a fragment of unknown origin; Lane 4, 13HE does not appear to contain any fragment; Lane 5, 12HE pBSK(+)$MG_{z\alpha}2$(7.0H/E); and Lane 6, 16HE pBSK(+)$MG_{z\alpha}2$(7.0H/E). The size marker used, λ/*Hin*dIII is reproduced on the left.

(B) Diagnostic Restriction Digests of the pBSK(+)$MG_{z\alpha}2$(7.0H/E) Clone, 10HE & **(C)** Corresponding Southern Analysis. An example of the restriction mapping used to further characterise each of the $MG_{z\alpha}2$(7.0HE) genomic subclones. The λ/*Hin*dIII size markers are reproduced on the left. The enzymes used to restrict the clone, include: Lane 1, *Kpn*I; Lane 2, *Kpn*I/*Bam*HI; Lane 3, *Bam*HI; Lane 4, *Xho*I; Lane 5, *Xho*I/*Bam*HI; Lane 6, *Xho*I/*Pst*I; Lane 7, *Pst*I; Lane 8, *Xba*I/*Pst*I; Lane 9, *Xba*I; Lane 10, *Xba*I/*Bam*HI; Lane 11, *Xba*I/*Sac*I; Lane 12, *Sac*I; and Lane 13, *Sal*I. DNA fragments to note in this figure include the : (i) 4kb *Kpn*I fragment in Lane 1; (ii) 2kb *Bam*HI fragment in Lane 3; and the 1.7kb *Sac*I fragment in Lane 12.

A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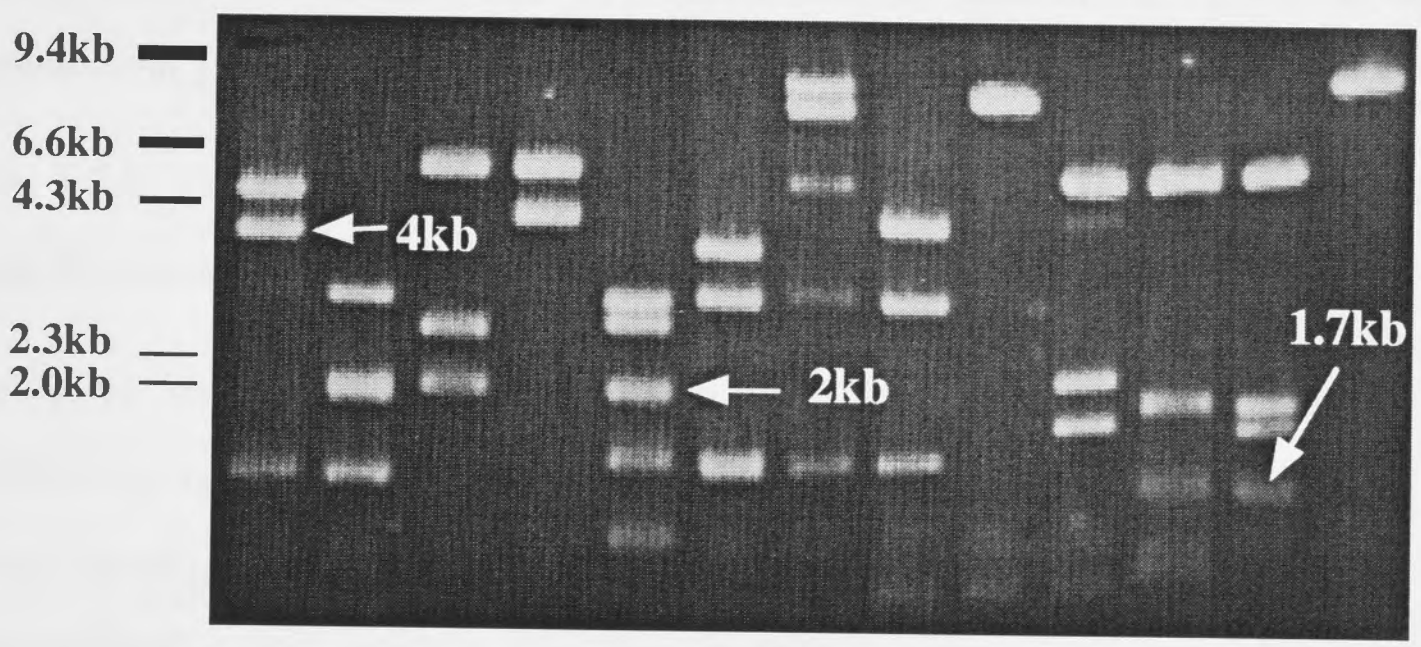
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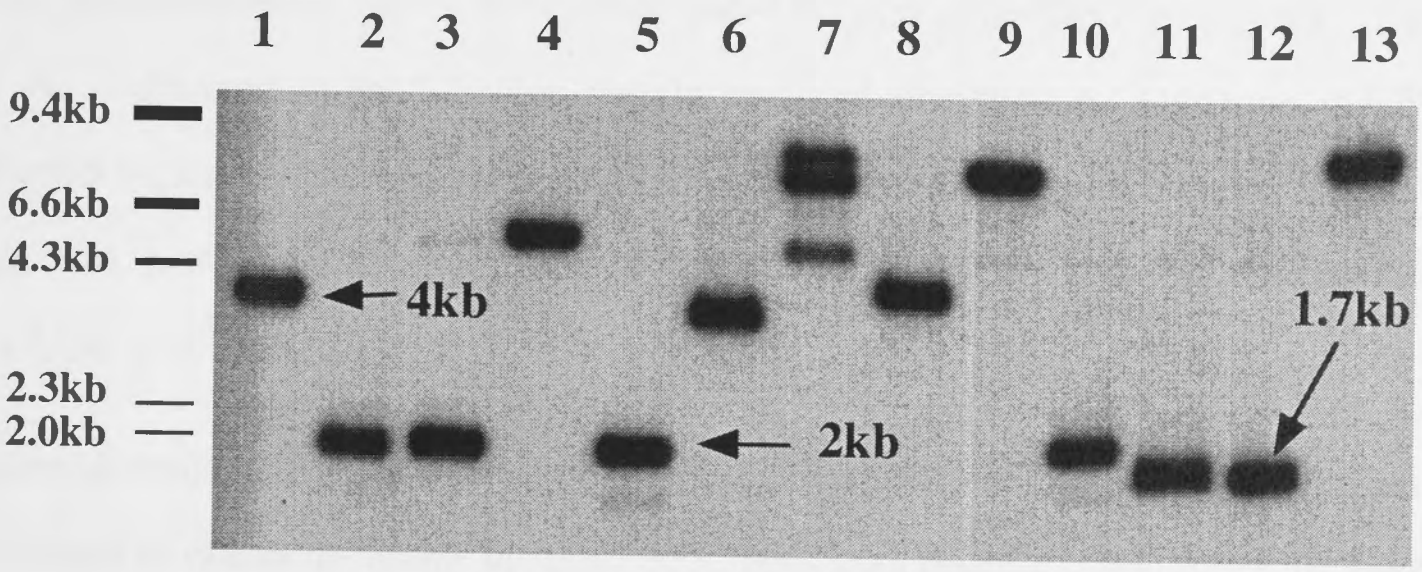
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prepared using the Wizard® Miniprep Purification System (Section 2.2.1, p32). These new clones were designated 6HE, 10HE, 12HE and 16HE respectively.

b) Restriction Mapping the $MG_{z\alpha}2$(7.0H/E) Genomic Fragment. To confirm that the correct $MG_{z\alpha}2$(7.0H/E) fragment had been isolated, the DNA from each of the pBSK(+)$MG_{z\alpha}2$(7.0H/E) clones was subjected to a series of enzyme digests and the DNA separated using agarose (1%) gel electrophoresis (Figure 3.7B). To further map the position of exon2, the DNA from these gels was then transferred to PCNM, using alkaline transfer and hybridised with endlabelled 1BF, under conditions of high stringency (63°C; Figure 3.7C). It was found that 1BF hybridised to a 4kb *Kpn*I [$MG_{z\alpha}2$(4.0K)], a 1.7kb *Sac*I [$MG_{z\alpha}2$(2.25S)] and a 2kb *Bam*HI [$MG_{z\alpha}2$(2.0B)] genomic fragment. Later sequencing would reveal that, although both *Sac*I and *Bam*HI restriction cut exon2, *Kpn*I does not (Section 2.1.1, p30; see below).

Section 3.2.5 Confirmation of $MG_{z\alpha}$ within the pBSK(+)$MG_{z\alpha}2$(7.0H/E) Genomic Fragment by DNA Sequencing.

a) Sequencing Strategy. Once the 1BF positive pBSK(+)$MG_{z\alpha}2$(7.0H/E) clone was obtained it was necessary to confirm the presence of the $MG_{z\alpha}$ exon2 and not an unknown homolog, before it could be confidently used to specifically disrupt the expression of $MG_{z\alpha}$. This would be done through DNA sequencing (Part 2.6, p52) using primers: (i) strategically placed within the coding region of $MG_{z\alpha}$ exon2, 1BF and 4BF; (ii) their 'complimentary' pairs, designated anti-1BF and anti-4BF respectively; (iii) mG_zE_1R, positioned at the start of translation, and designed to sequence the 5' untranslated region (UTR) of exon2; and (iv) the universal (M13) and reverse primer binding sites, positioned to either side of the pBSK(+) mutiple cloning site (MCS; Section 2.2.4, p36; Table 2.1).

b) Features Identified. The sequence data from each reaction (Part 2.6, p52) was gathered and combined to obtain the entire sequence of a large open reading frame (ORF, 723bp) later found to be highly similar in sequence and predicted protein product to both $RG_{z\alpha}$ and $HG_{z\alpha}$ exon2 (Figure 3.8 & 3.9). This sequencing also identified some of the endonuclease recognition sites, first recognised through restriction mapping (e.g. *Bam*HI

Figure 3.8 The $MG_{z\alpha}$, $RG_{z\alpha}$ and $HG_{z\alpha}$ Coding Sequences.

The cloning of $MG_{z\alpha}$ exon2 was confirmed, when the large ORF harboured by the putative $MG_{z\alpha}$2(7.0H/E) genomic clone was shown to have a high degree of sequence similarity with the $RG_{z\alpha}$ (95.9%, **R**) and $HG_{z\alpha}$ (92.9%, **H**) gene coding regions (Fong *et al.*, 1988; Matsuoka *et al.*, 1988). The figure below also is the completed sequence of the $MG_{z\alpha}$ exon2 coding region, obtained by combining the sequence of the partial $MG_{z\alpha}$ cDNA clone $MG_{z\alpha}$P (**P**; Leck, 1993), and the sequence of the 723bp ORF within the pBSK(+)$MG_{z\alpha}$2(7.0H/E) genomic clone (**M**). Note that the sequence of the putative $MG_{z\alpha}$ exon2 ends at the 'consensus' exon2/exon3 boundary (**V**).

```
          5'
M   1  ATG GGA TGT CGG CAA AGC TCA GAG GAA AAA GAG GCA GCG
P   -  --- --- --- --- --- --- --- --- --- --- --- --- ---
R   1  ATG GGA TGT CGG CAA AGC TCA GAG GAA AAA GAG GCA GCG
H   1  ATG GGA TGT CGG CAA AGC TCA GAG GAA AAA GAA GCA GCC

M  40  AGG CGG TCC CGG AGA ATT GAC CGC CAC CTG CGC TCC GAA
P   -  --- --- --- --- --- --- --- --- --- --- --- --- ---
R  40  CGG CGG TCG AGG AGA ATT GAC CGC CAC CTG CGC TCG GAG
H  40  CGG CGG TCC CGG AGA ATT GAC CGC CAC CTG CGC TCA GAA

M  79  AGC CAG CGG CAG CGC CGT GAA ATC AAA CTT CTC CTG CTG
P   -  --- --- --- --- --- --- --- --- --- --- --- --- ---
R  79  AGC CAG CGG CAG CGC CGT GAG ATC AAA CTT CTC CTG CTG
H  79  AGC CAG CGG CAA CGC CGC GAA ATC AAG CTG CTC CTG CTG

M 118  GGC ACC AGC AAC TCG GGC AAG AGC ACC ATC GTC AAG CAG
P   -  --- --- --- --- --- --- --- --- --- --- --- --- ---
R 118  GGC ACC AGC AAC TCG GGC AAG AGC ACC ATC GTC AAG CAG
H 118  GGC ACC AGC AAC TCA GGC AAG AGC ACC ATC GTC AAA CAG

M 157  ATG AAG ATC ATC CAC AGC GGG GGC TTC AAC CTG GAC GCC
P   -  --- --- --- --- --- --- --- --- --- --- --- --- ---
R 157  ATG AAA ATC ATC CAC AGC GGT GGT TTC AAC CTG GAG GCC
H 157  ATG AAG ATC ATC CAC AGC GGC GGC TTC AAC CTG GAG GCC

M 196  TGC AAG GAG TAC AAG CCC CTC ATC ATC TAC AAC GCC ATC
P   -  --- --- --- --- --- --- --- --- --- --- --- --- ---
R 196  TGC AAG GAG TAC AAG CCC CTC ATC ATC TAC AAC GCC ATC
H 196  TGC AAG GAG TAC AAG CCC CTC ATC ATC TAC AAT GCC ATC

M 235  GAC TCG CTG ACC CGG ATC ATC CGG GCC CTG GCT GCC CTC
P   -  --- --- --- --- --- --- --- --- --- --- --- --- ---
R 235  GAC TCG CTG ACC CGG ATC ATT CGG GCC CTG GCT GCC CTG
H 235  GAC TCG CTG ACC CGG ATC ATC CGG GCC CTG GCC GCC CTC

M 274  AAG ATC GAT TTC CAC AAC CCT GAC CGT GCC TAC GAC GCT
P   -  --- --- --- --- --- --- --- --- --- --- --- --- ---
R 274  AAG ATT GAT TTC CAC AAC CCT GAC CGT GCC TAC GAC GCC
H 274  AGG ATC GAC TTC CAC AAC CCC GAC CGC GCC TAC GAC GCT
```

```
M 313 GTG CAG CTC TTT GCT CTG ACT GGC CCA GCA GAG AGC AAG
P  -  --- --- --- --- --- --- --- --- --- --- --- --- ---
R 313 GTG CAG CTC TTT GCC CTG ACT GGC CCG GCA GAG AGC AAG
H 313 GTG CAG CTC TTT GCG CTG ACG GGC CCC GCT GAG AGC AAG

                                    ____________4BF____________
M 352 GGT GAG ATT ACA CCT GAG CTG CTG GGT GTC ATG CGA CGG
P 1   --- --- --- --- --- --- --- --- --- --- --- CGA CGG
R 352 GGT GAG ATC ACG CCC GAG CTG CTG GGT GTC ATG CGA CGG
H 352 GGC GAG ATC ACA CCC GAG CTG CTG GGT GTC ATG CGA CGG

M 391 CTC TGG GCT GAC CCA GGG GCC CAG GCC TGC TTT GGC CGC
P 7   CTC TGG GCT GAC CCA GGG GCC CAG GCC TGC TTT GGC CGC
R 391 CTC TGG GCT GAC CCC GGG GCC CAG GCC TGC TTC GGC CGC
H 391 CTC TGG GCC GAC CCA GGG GCA CAG GCC TGC TTC AGC CGC

M 430 TCT AGC GAG TAC CAC CTG GAG GAC AAT GCA GCC TAC TAC
P  46 TCT AGC GAG TAC CAC CTG GAG GAC AAT GCA GCC TAC TAC
R 430 TCC AGC GAG TAC CAC CTG GAG GAC AAC GCC GCT TAC TAC
H 430 TCC AGC GAG TAC CAC CTG GAG GAC AAC GCG GCC TAC TAC

M 469 CTG AAC GAC CTG GAG CGC ATC GCA GCG CCC GAC TAC ATC
P  85 CTG AAC GAC CTG GAG CGC ATC GCA GCG CCC GAC TAC ATC
R 469 CTG AAT GAC CTG GAG CGC ATC GCG GCG CCC GAC TAT ATC
H 469 CTG AAC GAC CTG GAG CGC ATC GCC GCA GCT GAC TAT ATC

M 508 CCC ACG GTG GAG GAT ATC CTA CGC TCC CGG GAC ATG ACC
P 124 CCC ACG GTG GAG GAT ATC CTA CGC TCC CGG GAC ATG ACC
R 508 CCC ACG GTG GAG GAC ATC CTG CGC TCT CGG GAC ATG ACC
H 508 CCC ACT GTC GAG GAC ATC CTG CGC TCC CGG GAC ATG ACC

M 547 ACG GGC ATT GTG GAG AAC AAG TTC ACC TTC AAG GAG CTT
P 163 ACG GGC ATT GTG GAG AAC AAG TTC ACC TTC AAG GAG CTT
R 547 ACG GGC ATT GTG GAA AAC AAG TTC ACC TTC AAG GAG CTT
H 547 ACG GGC ATT GTG GAG AAC AAG TTC ACC TTC AAG GAG CTC

M 586 ACC TTC AAG ATG GTG GAC GTG GGC GGG CAG AGG TCA GAA
P 202 ACC TTC AAG ATG GTG GAC GTG GGC GGG CAG AGG TCA GAA
R 586 ACC TTC AAG ATG GTG GAT GTG GGA GGG CAG AGG TCA GAG
H 586 ACC TTC AAG ATG GTG GAC GTG GGG GGG CAG AGG TCA GAG

M 625 CGC AAA AAG TGG ATC CAT TGC TTT GAA GGC GTC ACA GCC
P 241 CGC AAA AAG TGG ATC CAT TGC TTT GAA GGC GTC ACA GCC
R 625 CGC AAA AAA TGG ATC CAC TGC TTT GAG GGC GTG ACG GCC
H 625 CGC AAA AAG TGG ATC CAC TGC TTC GAG GGC GTC ACA GCC

M 664 ATC ATC TTC TGT GTG GAG CTC AGT GGC TAT GAC CTG AAG
P 280 ATC ATC TTC TGT GTG GAG CTC AGT GGC TAT GAC CTG AAG
R 664 ATC ATC TTC TGT GTG GAG CTC AGT GGC TAT GAC CTG AAG
H 664 ATC ATC TTC TGT GTG GAG CTC AGC GGC TAC GAC CTG AAA

                                                V
M 703 CTC TAT GAG GAC AAC CAG ACG --- --- --- --- --- ---
P 319 CTC TAT GAG GAC AAC CAG ACG AGC CGG ATG GCG GAG AGC
R 703 CTT TAT GAG GAC AAC CAG ACG AGC CGA ATG GCG GAG AGC
H 703 CTC TAC GAG GAT AAC CAG ACA AGT CGG ATG GCA GAG AGC
```

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M   -  --- --- --- --- --- --- --- --- --- --- --- --- ---
P 358 CTG CGC CTC TTT GAC TCC ATC TGC AAC AAC AAC TGG TTC
R 742 CTG CGT CTT TTT GAC TCC ATC TGC AAC AAC AAC TGG TTC
H 742 TTG CGC CTC TTT GAC TCC ATC TGC AAC AAC AAC TGG TTC

M   -  --- --- --- --- --- --- --- --- --- --- --- --- ---
P 397 ATC AAC ACC TCG CTC ATC CTC TTC CTG AAC AAG AAG GAC
R 781 ATC AAC ACC TCC CTC ATC CTC TTC CTG AAC AAG AAG GAC
H 781 ATC AAC ACC TCA CTC ATC CTC TTC CTG AAC AAG AAG GAC

M   -  --- --- --- --- --- --- --- --- --- --- --- --- ---
P 436 CTC CTG GCA GAG AAG ATC CGG CGT ATC CCG CTC AGC GTC
R 820 CTC CTG TCG GAG AAG ATT CGG CGT ATC CCG CTC AGC GTC
H 820 CTG CTG GCA GAG AAG ATC CGC CGC ATC CCG CTC ACC ATC

M   -  --- --- --- --- --- --- --- --- --- --- --- --- ---
P 475 TGC TTC CCA GAG TAC AAG GGT CAG AAC ACG TAC GAG GAA
R 859 TGC TTC CCC GAG TAC AAG GGT CAG AAC ACG TAC GAG GAA
H 859 TGC TTT CCC GAG TAC AAG GGC CAG AAC ACG TAC GAG GAG

M   -  --- --- --- --- --- --- --- --- --- --- --- --- ---
P 514 GCC GCG GTC TAC ATC CAA CGT CAG TTC GAG GAC CTC AAC
R 898 GCC GCG GTC TAC ATC CAG CGT CAG TTC GAG GAC CTA AAC
H 898 GCC GCT GTC TAC ATC CAG CGG CAG TTT GAA GAC CTG AAC

M   -  --- --- --- --- --- --- --- --- --- --- --- --- ---
P 553 CGC AAC AAG GAG ACC AAG GAG ATC TAT TCG CAC TTC ACT
R 937 CGA AAC AAG GAG ACC AAG GAG ATC TAC TCG CAC TTT ACC
H 937 CGC AAC AAG GAG ACC AAG GAG ATC TAC TCC CAC TTC ACC

M   -  --- --- --- --- --- --- --- --- --- --- --- --- ---
P 592 TGT GCC ACC GAC ACC AGT AAC ATC CAG TTT GTG TTT GAC
R 976 TGT GCC ACC GAC ACC AGT AAC ATC CAG TTT GTC TTC GAC
H 976 TGC GCC ACC GAC ACC AGT AAC ATC CAG TTT GTC TTC GAC

                                              3R
M   -  --- --- --- --- --- --- --- --- --- --- --- --- ---
P 631 GCA GTG ACA GAT GTC ATC ATA CAG AAC AAT CTC AAG TAC
R1015 GCA GTG ACA GAT GTC ATC ATA CAG AAC AAT CTC AAG TAC
H1015 GCG GTG ACA GAC GTC ATC ATA CAG AAC AAT CTC AAG TAC

      ---------- 3'
M     --- --- --- --- ---
P     ATT GGC --- --- ---
R1059 ATT GGC CTT TGC TGA
H1059 ATT GGC CTT TGC TGA
```

Figure 3.9 The Predicted $MG_{z\alpha}$, $RG_{z\alpha}$ and $HG_{z\alpha}$ Amino Acid Sequences.

A comparison of the completed $MG_{z\alpha}$ (**M**), $RG_{z\alpha}$ (**R**) and $HG_{z\alpha}$ (**H**) predicted peptide sequences shows that there is little difference between them, and none at all in the sequence upstream of the partial cDNA clone $MG_{z\alpha}P$ (flanked by 4BF & 3R). This provides compelling evidence that the $MG_{z\alpha}2$(7.0H/E) genomic fragment contains at least part of the native $MG_{z\alpha}$ gene. Note that the putative mouse sequence differs from the rat by only one amino acid, at position 275 (*; Leck, 1993).

```
M1     MGCRQSSEEK EAARRSRRIN RHLRSESQRQ RREIKLLLLG TSNSGKSTIV
R1     MGCRQSSEEK EAARRSRRIN RHLRSESQRQ RREIKLLLLG TSNSGKSTIV
H1     MGCRQSSEEK EAARRSRRIN RHLRSESQRQ RREIKLLLLG TSNSGKSTIV

M51    KQMKIIHSGG FNLEACKEYK PLIIYNAIDS LTRIIRALAA LRIDFHNPDR
R51    KQMKIIHSGG FNLEACKEYK PLIIYNAIDS LTRIIRALAA LRIDFHNPDR
H51    KQMKIIHSGG FNLEACKEYK PLIIYNAIDS LTRIIRALAA LRIDFHNPDR

                                    ____4BF__
M101   ATDAVQLFAL TGPAESKGEI PELLGVMRRL WADPGAQACF GRSSEYHLED
R101   ATDAVQLFAL TGPAESKGEI PELLGVMRRL WADPGAQACF GRSSEYHLED
H101   ATDAVQLFAL TGPAESKGEI PELLGVMRRL WADPGAQACF GRSSEYHLED

M151   NAAYYLNDLE RIAAPDYIPT VEDILRSRDM TTGIVENKFT FKELTFKMVD
R151   NAAYYLNDLE RIAAPDYIPT VEDILRSRDM TTGIVENKFT FKELTFKMVD
H151   NAAYYLNDLE RIAAADYIPT VEDILRSRDM TTGIVENKFT FKELTFKMVD

M201   VGGQRSERKK WIHCFEGVTA IIFCVELSGY DLKLYEDNQT SRMAESLRLF
R201   VGGQRSERKK WIHCFEGVTA IIFCVELSGY DLKLYEDNQT SRMAESLRLF
H201   VGGQRSERKK WIHCFEGVTA IIFCVELSAY DLKLYEDNQT SRMAESLRLF

                                       *
M251   DSICNNNWFI NTSLILFLNK KDLLAEKIRR IPLSVCFPEY KGQNTYEEAA
R251   DSICNNNWFI NTSLILFLNK KDLLSEKIRR IPLSVCFPEY KGQNTYEEAA
H251   DSICNNNWFI NTSLILFLNK KDLLAEKIRR IPLTICFPEY KGQNTYEEAA

                                                      ____3R__
M301   VYIQRQFEDL NRNKETKEIY SHFTCATDTS NIQFVFDAVT DVIIQNNLKY
R301   VYIQRQFEDL NRNKETKEIY SHFTCATDTS NIQFVFDAVT DVIIQNNLKY
H301   VYIQRQFEDL NRNKETKEIY SHFTCATDTS NIQFVFDAVT DVIIQNNLKY

       __
M351   IG-----
R351   IGLCend
H351   IGLCend
```

Figure 3.10 The $MG_{z\alpha}$ Cloning Strategy.

(A) A restriction map of the $\lambda MG_{z\alpha}1$ genomic clone, obtained through high stringency hybridisation with radiolabelled 1BF (Figure 3.6). Note the 2kb *Bam*HI genomic fragment within the larger genomic 7kb *Hin*dIII/*Eco*RI fragment.

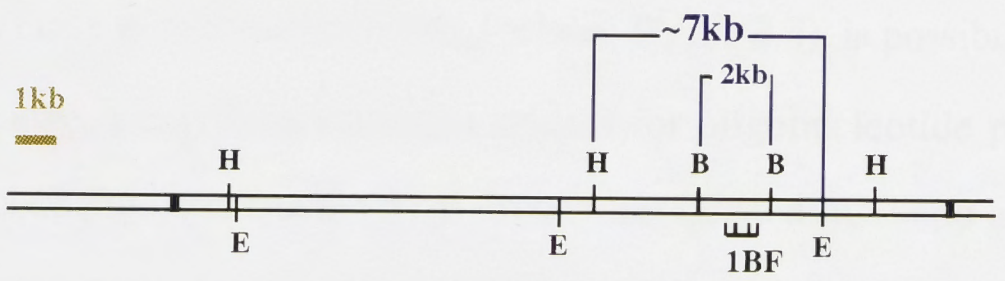
(B) Map of the $MG_{z\alpha}2(7.0H/E)$ genomic subclone (Figure 3.7). Also shown in this figure is the position of the $MG_{z\alpha}2(4.0K)$ fragment, which encompasses both the coding and non-coding regions of exon2, as well as a number of the restriction sites (*), confirmed through preliminary sequencing. The approximate region sequenced and the position of each of the primers is marked: 1BF (3); anti-1BF (2); 4BF/4CF (4); anti-4BF/anti-4CF (1); and mG_zE_1R (5). Both the reverse and universal (M13) primers are marked.

(C) A diagramatic representation of the $pBKS(-)MG_{z\alpha}2(4.0K)$ subclone. The *Bam*HI restriction pattern will change with respect to the MCS of the cloning vector, a property which would later be used to identify both orientations of the cloned insert (Figure 3.11).

Abbreviations: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; S, *Sac*I; Sa, *Sal*I; X, *Xba*I & Xh, *Xho*I.

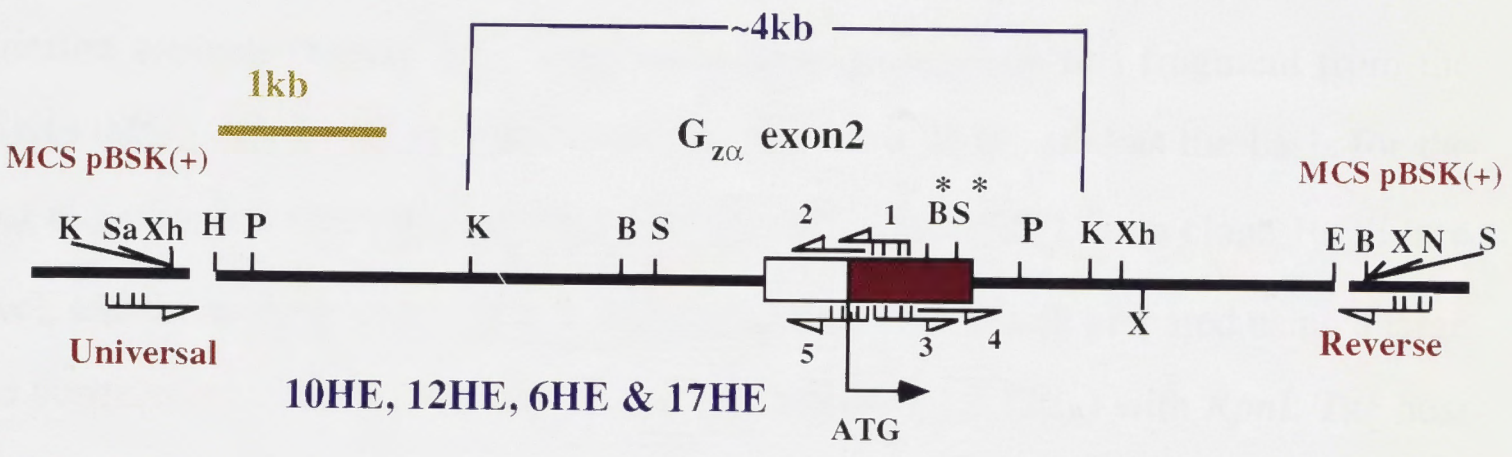
A

λ Phage Genomic Clone λMG$_{z\alpha}$1



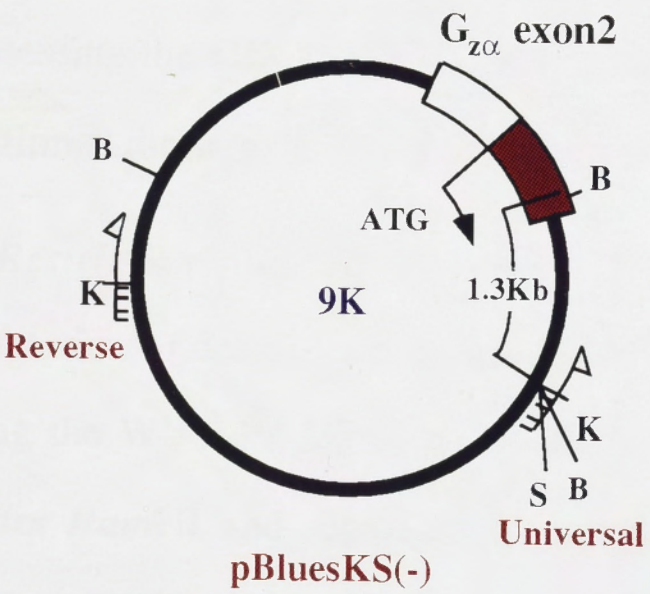
B

pBSK(+)MG$_{z\alpha}$ 2(7.0HE)

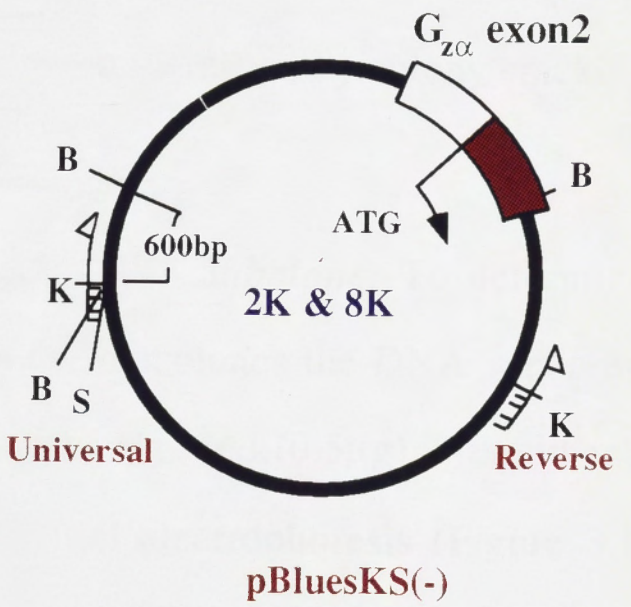


C

pBKS(-) MG$_{z\alpha}$ 2(4.0K) Ori I



pBKS(-) MG$_{z\alpha}$ 2(4.0K) Ori II



& *Sac*I; Figure 3.7) and a single base pair difference between the 4BF oligonucleotide and the corresponding mouse genomic sequence. C-366 ($RG_{z\alpha}$ & $HG_{z\alpha}$) is substituted for T-366 in the $MG_{z\alpha}$ gene (Figure 3.8). Although this change has no affect on the translated protein it did, however, put into question the specificity of the 4BF primer. That 4BF had worked so far (e.g. to create the $MG_{z\alpha}P$ clone; Figure 3.5), is possibly due to the 5' nature of the change, a region not thought critical for oligonucleotide priming. To compensate for this change a corrected 4BF primer, designated 4CF and an anti-4CF primer (Table 2.1) were designed and used in place of 4BF/anti-4BF, when necessary.

Section 3.2.6 Cloning & Characterising the 4kb *Kpn*I $MG_{z\alpha}$ Subclone.

a) Isolation of the pBKS(-)$MG_{z\alpha}$2(4.0K) Fragment. Because the entire coding region of exon2 lay within the 1BF positive $MG_{z\alpha}$2(4.0K) fragment, previously identified through restriction analysis (Figure 3.7), steps were taken to separate this fragment from the pBSK(+)$MG_{z\alpha}$2(7.0H/E) genomic clone, so that it could be used as the basis for the $MG_{z\alpha}$ targeting construct. The plasmid pBSK(+)$MG_{z\alpha}$2(7.0H/E), from clone 10HE (see above), and the cloning vector pBKS(-) (Appendix 2.1) were both prepared using a large scale purification method (Section 2.2.1, p32) and restricted (2µg) with *Kpn*I. The host vector was then dephosphorylated (Section 2.3.5, p39) and separated from uncut vector using agarose (1%) gel electrophoresis. This was repeated to isolate the $MG_{z\alpha}$2(4.0K) fragment, from the rest of the pBSK(+)$MG_{z\alpha}$2(7.0H/E) clone and both 'DNA bands' were purified by freeze squeezing (Section 2.3.3, p38). Following a 2hr ligation at room temperature, and transformation into the chemically competent *E. coli* DH5α, those clones possessing the pBKS(-)$MG_{z\alpha}$2(4.0K) construct were identified by colony cracking and confirmed through the use of restriction analysis.

b) Restriction Analysis of the pBKS(-)$MG_{z\alpha}$2(4.0K) Subclone. To determine the orientation of each of these putative $MG_{z\alpha}$2(4.0K) subclones the DNA was prepared using the Wizard® Miniprep Purification System, digested (0.5µg) with either *Kpn*I and/or *Bam*HI and separated by agarose (1%) gel electrophoresis (Figure 3.11A). Designated 2K, 8K and 9K, each clone was found to represent both orientations of the $MG_{z\alpha}$2(4.0K) fragment. Clones 2K and 8K, OriII and Clone 9K, OriI (Figure 3.10B).

Figure 3.11 The Characterisation of the $MG_{z\alpha}2$(4.0K) Subclones.

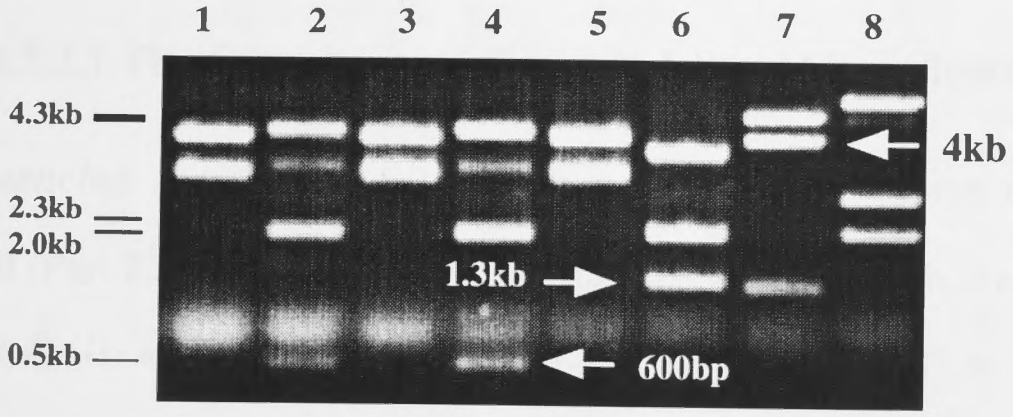
(A) Diagnostic analysis of the pBSK(+)$MG_{z\alpha}2$(4.0K) subclones. Lane 1, 2K plasmid DNA digested with *Kpn*I (2K/*Kpn*I); Lane 2, 2K/*Bam*HI; Lane 3, 8K/*Kpn*I; Lane 4, 8K/*Bam*HI; Lane 5, 9K/*Kpn*I; Lane 6, 9K/*Bam*HI; Lanes 7 & 8 contain the original 6HE pBSK(+)$MG_{z\alpha}2$(7.0HE) clone cut with *Kpn*I and *Bam*HI respectively. The size marker (λ/*Hin*dIII) is reproduced on the left . The gel clearly shows (Lanes 1, 3 & 5) that each of these new constructs possess a 4kb band which corresponds to the same $MG_{z\alpha}2$(4.0K) fragment cut from pBSK(+)$MG_{z\alpha}2$(7.0H/E) (Lane 7). The smaller 2.9kb band represents the linearised host vector pBKS(-), whilst the 2kb *Bam*HI band (Lanes 2, 4 & 6), corresponds to the fragment identified through earlier restriction mapping of the $\lambda MG_{z\alpha}$ genomic clones and the $MG_{z\alpha}2$(7.0H/E) genomic subclone (Figure 3.10). Therefore, the *Bam*HI restriction fragment pattern for OriI: 3.6kb, 2kb & 1.3kb (Lane 6); and for OriII: 4.3kb, 2kb and 0.6kb (Lanes 2 & 4).

(B) Shown in the figure is a schematic representation of the strategy chosen to sequence both orientations of the $MG_{z\alpha}2$(4.0K) cloned fragment.

(C) The figure shown is an example of the band pattern following timed deletion of the $MG_{z\alpha}2$(4.0K) insert within clone 9K (OriI). Lane 1 contains 250ng of linear pBKS() $MG_{z\alpha}2$(4.0K). Lanes 2-16 contains a sample of each of the timed aliquot's, following *Exo*III degradation. On the far right is the approximate position of linear pBKS(-) vector (2.9kb). The arrow indicates the residual 'undigested' or 'undeleted' pBKS() $MG_{z\alpha}2$(4.0K) construct (6.9kb).

Abbreviations: E, *Eco*RI; K, *Kpn*I & N, *Not*I.

A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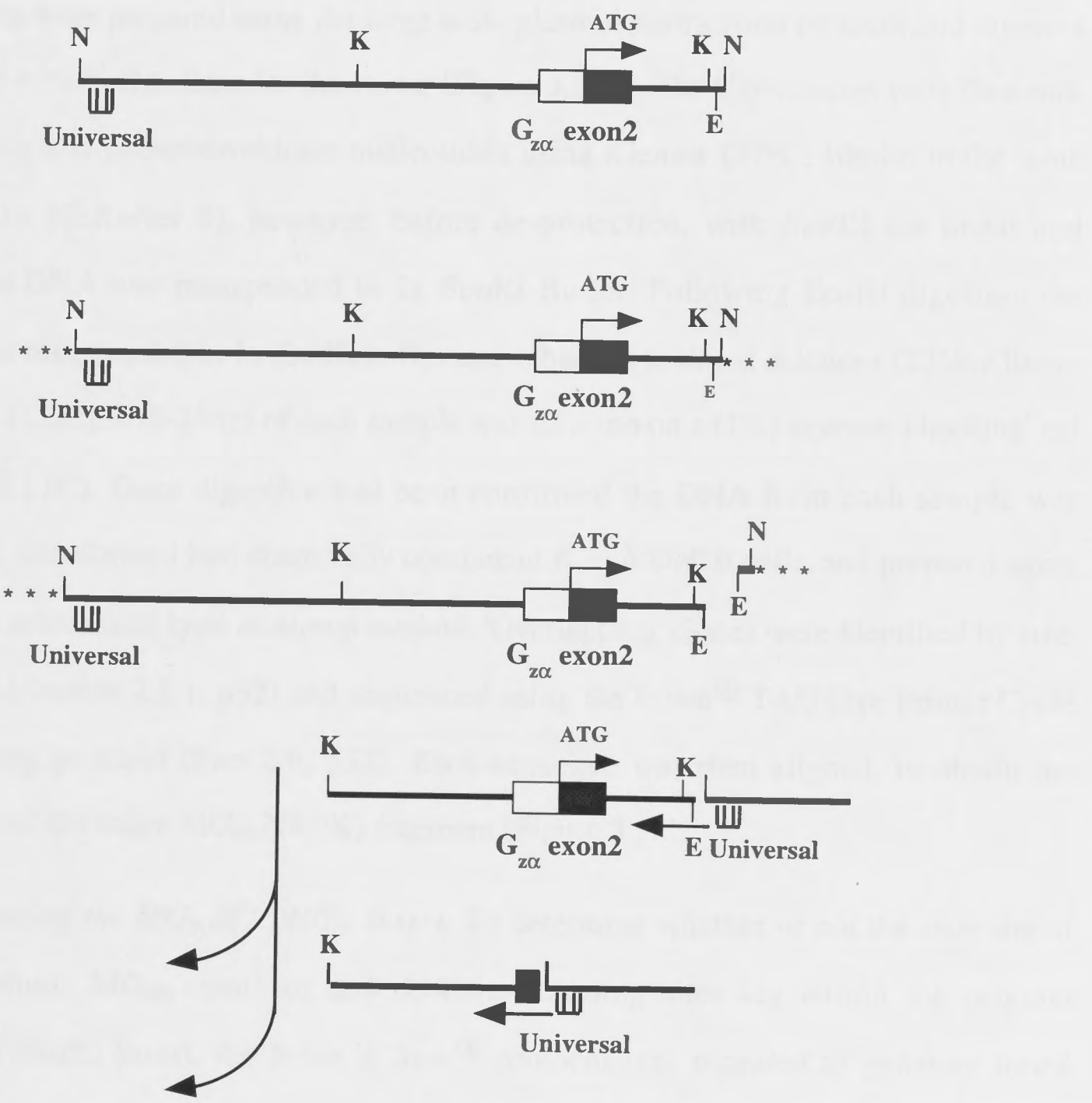
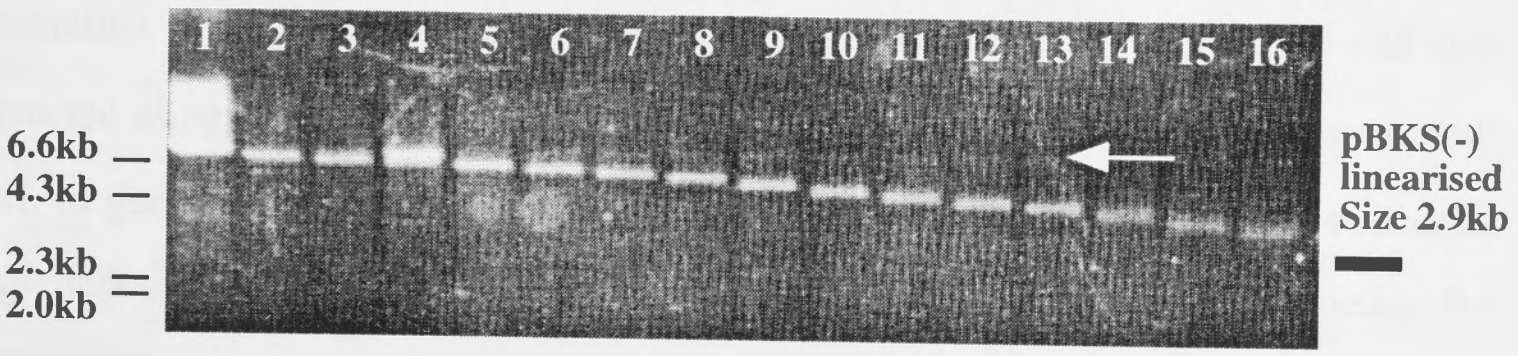
4.3kb
2.3kb
2.0kb
0.5kb
1.3kb
600bp
4kb

B


N
K
ATG
K N
Universal
$G_{z\alpha}$ exon2
E
N
K
ATG
K N
Universal
$G_{z\alpha}$exon2
E
N
K
ATG
K
N
E
Universal
$G_{z\alpha}$ exon2
E
K
ATG
K
$G_{z\alpha}$ exon2
E Universal
K
Universal

C


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
6.6kb
4.3kb
2.3kb
2.0kb
pBKS(-)
linearised
Size 2.9kb

Section 3.2.7 The Generation of Timed Deletion $MG_{z\alpha}$ Clones.

a) Sequencing the $MG_{z\alpha}2(4.0K)$ Insert. A modified version of the Erase a Base® Protocol (Part 2.5, p48) was used to completely sequence both orientations/strands (OriI, sense & OriII, anti-sense) of the $MG_{z\alpha}2(4.0K)$ cloned insert so that endogenous PCR primers could be confidently designed and later used to screen $MG_{z\alpha}$ targeted ES cells *in vitro* (Part 3.1, p56). Both the 9K (OriI) and 8K (OriII) pBKS(-)$MG_{z\alpha}2(4.0K)$ constructs were prepared using the large scale plasmid purification protocol and digested (5-10μg) with *Not*I to linearise the vector (Figure 3.11B). The 5' overhangs were then end-filled with α-α-phosphorothioate nucleotide's using Klenow (37°C, 10min) in the same buffer (1x NEBuffer 3), however, before de-protection, with *Eco*RI the linear and protected DNA was resuspended in 1x *Eco*RI Buffer. Following *Eco*RI digestion the DNA was resuspended in 1x *Exo*III buffer and subjected to timed deletions (225bp/30sec; Figure 2.1). 2.5μL (5-25ng) of each sample was then run on a (1%) agarose 'checking' gel (Figure 3.11C). Once digestion had been confirmed the DNA from each sample was religated, transformed into chemically competent *E. coli* DH5α cells, and prepared using the small scale alkali lysis miniprep method. 'Overlapping' clones were identified by size, prepared (Section 2.2.1, p32) and sequenced using the Prism® TAQ Dye Primer Cycle Sequencing protocol (Part 2.6, p52). Each sequence was then aligned, to obtain the sequence of the entire $MG_{z\alpha}2(4.0K)$ fragment (Figure 3.12).

b) Sequencing the $MG_{z\alpha}2(7.0H/E)$ Insert. To determine whether or not the start site of transcription, $MG_{z\alpha}$ exon1 or any promoter binding sites lay within the original $MG_{z\alpha}2(7.0H/E)$ insert, the Erase a Base® protocol was repeated to generate timed deletions of the 6HE, pBSK(+)$MG_{z\alpha}2(7.0H/E)$ construct (Figure 3.10B). In this instance using *Not*I and *Eco*RI the 'reverse' primer was protected, however, because only one orientation of the $MG_{z\alpha}2(7.0H/E)$ insert was available, the universal primer was also protected using a second pair of enzymes in order to sequence the complimentary strand: *Sal*I, to generate the primer protection site, and *Hin*dIII, to expose one end to *Exo*III degradation. This sequencing helped to confirm that like the $RG_{z\alpha}$ and $HG_{z\alpha}$ genes, the $MG_{z\alpha}$ exon1, does not lie in close proximity to exon2 (Figure 3.5B), but may, like the second exon, be present on some other λM genomic clone.

Figure 3.12 The Complete Sequence of the $MG_{z\alpha}2$(4.0K) Subclone.

The sequence from each of the deletion clones (both strands) were combined to obtain the complete consensus sequence of the $MG_{z\alpha}2$(4.0K) subclone (actually 3 888bp). This shows that the entire $MG_{z\alpha}$ second exon (underlined), including both the coding and non-coding regions, has been subcloned. Points of interest to note are: (i) the putative splice acceptor which corresponds to the exon1/exon2 boundary (TTCTCTCTTTCCTTAG; Table 3.2); (ii) the *Bsa*BI (2095bp-2104bp) recognition sequence within the coding region and positioned close to the start site of translation; (iii) the *Acc*I (3803-3808) restriction site at the 5' end of the clone; and (iv) the primer binding sites mG_zE_1R, mG_zG_F, mG_zG_R, mG_zG_F(*Not*I), 1BF, 4BF, Al-1 and Al-2 (Table 2.1). The primers 1BF, 4BF and G_zE_1R were used to clone the exon and provide preliminary sequence data (Figure 3.8; 3.9), whilst the rest would later be used later.

5'

```
                    20                  40                  60
                    *                   *                   *
CTGACAAAAGGCCGTGTGAA GACCAGGATTTACACCCTGA  GAGCATCAGGTGGTGGCAAG

                    80                  100                 120
                    *                   *                   *
CGTGGACTCAGGGAGAGAGG TGGCTTCAGAGATACTGTTG GAGAAGGTGGGCCCAAAGGG

                   140                 160                 180
                    *                   *                   *
TTGGAGAAAACCAGAGGCCA TGGCCACTGGAGAAAGTCTG ATTTGTGGGCCCTGGGGCTT

                   200                 220                 240
                    *                   *                   *
GGTTCCAAGCAAGGCCTGTA ATGGCCAGCATGGGGGATGG GCTGCCACTTCCTGAGGGTC

                   260                 280                 300
                    *                   *                   *
CAAGTCAGATGTATAAAGAG TAACGTCGGTAGTTGGGTAG GCTGAGTTTCAGGTGCTGGT

                   320                 340                 360
                    *                   *                   *
TACACCTAGGAGTGGAGGTG GAGAGAGGCCCTTGCCTGTG TTAGAAACTTGTCTGCCTTG

                   380                 400                 420
                    *                   *                   *
GGCCTTCCATCCCAGAGCCT GGTATCAGGGAAGGGGACAT TAGATGGGTTTTAAAGTACC

                   440                 460                 480
                    *                   *                   *
CGAGGCTCAATAGCCAGGCA AAGACATACCAGAGGAAAGA AAGAAGGCAAGAGATGGGCC
```

```
                    500                 520                 540
                     *                   *                   *
ATGGTGTGGGGGTGGGGCAC CCTGGGCTCCCAGGCAGAGG CAGAACTGAGCCCCCTGCTG

                    560                 580                 600
                     *                   *                   *
GAGGAGACATGCCCCAGCCA CCCAGAGGGTCATGTTGAGA TCTAGGGGGTAACCTTGCCC

                    620                 640                 660
                     *                   *                   *
GTGGTAGGCAACAGGTGTCA CTGGATCCAGCTGTCAATAC AGCTGTTCTCCATTAACGCT

                    680                 700                 720
                     *                   *                   *
CAGGCCCACAGAGAGCCTTC GAGAAAGACTCACCCTGAGC CCTTCATAGGACCTGAGCAC

                    740                 760                 780
                     *                   *                   *
AGCAACCACACCTGGACTCT GAGTCCCAGTTTTCTGAGAG CCTCCCCACCGTTTCTTGCC

                    800                 820                 840
                     *                   *                   *
TCCCTAGACAGGTTATCTCG GTATTGCCCTGATTTCTCAG GCCTTGGCCATCCCCCAAGA

                    860                 880                 900
                     *                   *                   *
CCATTCCTGTGCCGAGCTTT TCGTGGCGCAAGACTCCATG GTGCCCCCATCGACTGATTC

                    920                 940                 960
                     *                   *                   *
CCCCTGGAAGGGACATCAGG GCCAGAGCTCTATCCTGACC CTGCTCTAAACATGCATCTG

                    980                 1000                1020
                     *                   *                   *
GCCCTAGGACGAGAGAGATG GTGGAAGTCAGGAGATACAC AGTTTTCCCTGGCCTTCTTC

                    1040                1060                1080
                     *                   *                   *
AGGCAGGGCCACAGCCAGGA CTGTGCTGTGAAGTCAAGTG GCACTGAGCCCCCTCCCTGT

                    1100                1120                1140
                     *                   *                   *
CCTGGGGGAGCGGACACCCA CAGCAGGGTTGTACTGTGGA GCCCCAGTCAGACAGACAGA

                    1160                1180                1200
                     *                   *                   *
TTCAGAGGGGCAGAGTCAGC CGGGTGTAGTAGGCAGGAAG CGGTGGTAGAGGTGGACGCA

                    1220                1240                1260
                     *                   *                   *
GCGTGGGGTCAGCGGGCCAG AGCCTTGCACAACTCAAGGT CTGCCCTGCACCTGGTCTCA

                    1280                1300                1320
                     *                   *                   *
AGCAGATTGCAGAGTTGAAG GCATCGGCCAAGCATATTTT TTTTGCTGTGGGCTGGGCCC

                    1340                1360                1380
                     *                   *                   *
TCACCCTGAAGCACTTAAAG GTCTGGGAGTTGCTGAACCG TAGTGCTGTTTTCTGAGGCC
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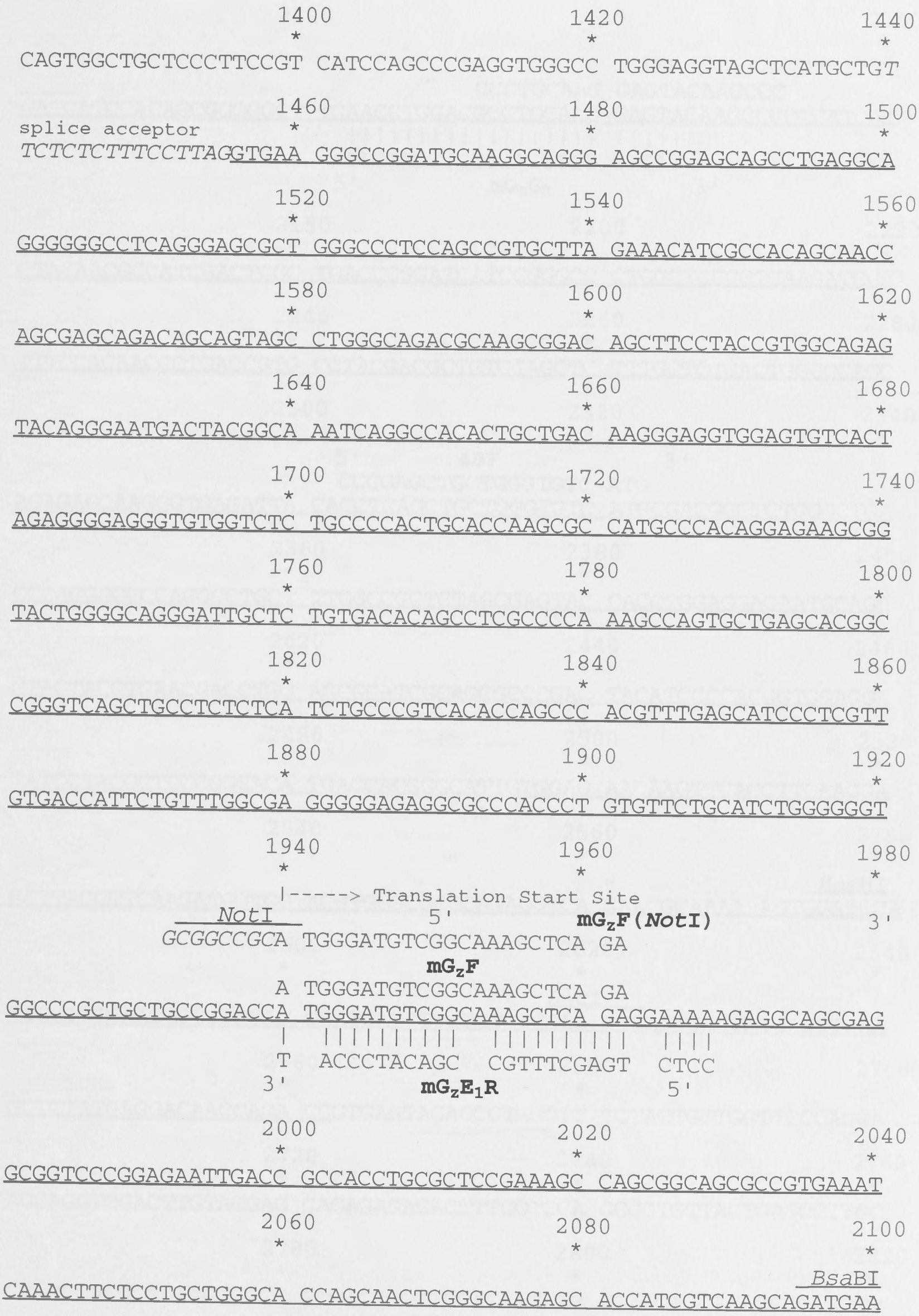
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1420
1440
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1460
1480
1500
splice acceptor
TCTCTCTTTCCTTAGGTGAA GGGCCGGATGCAAGGCAGGG AGCCGGAGCAGCCTGAGGCA
1520
1540
1560
GGGGGGCCTCAGGGAGCGCT GGGCCCTCCAGCCGTGCTTA GAAACATCGCCACAGCAACC
1580
1600
1620
AGCGAGCAGACAGCAGTAGC CTGGGCAGACGCAAGCGGAC AGCTTCCTACCGTGGCAGAG
1640
1660
1680
TACAGGGAATGACTACGGCA AATCAGGCCACACTGCTGAC AAGGGAGGTGGAGTGTCACT
1700
1720
1740
AGAGGGGAGGGTGTGGTCTC TGCCCCACTGCACCAAGCGC CATGCCCACAGGAGAAGCGG
1760
1780
1800
TACTGGGGCAGGGATTGCTC TGTGACACAGCCTCGCCCCA AAGCCAGTGCTGAGCACGGC
1820
1840
1860
CGGGTCAGCTGCCTCTCTCA TCTGCCCGTCACACCAGCCC ACGTTTGAGCATCCCTCGTT
1880
1900
1920
GTGACCATTCTGTTTGGCGA GGGGGAGAGGCGCCCACCCT GTGTTCTGCATCTGGGGGGT
1940
1960
1980
|-----> Translation Start Site
NotI
5'
mGzF(NotI)
3'
GCGGCCGCA TGGGATGTCGGCAAAGCTCA GA
mGzF
A TGGGATGTCGGCAAAGCTCA GA
GGCCCGCTGCTGCCGGACCA TGGGATGTCGGCAAAGCTCA GAGGAAAAAGAGGCAGCGAG
| |||||||||| |||||||||| ||||
T ACCCTACAGC CGTTTCGAGT CTCC
3'
mGzE1R
5'
2000
2020
2040
GCGGTCCCGGAGAATTGACC GCCACCTGCGCTCCGAAAGC CAGCGGCAGCGCCGTGAAAT
2060
2080
2100
BsaBI
CAAACTTCTCCTGCTGGGCA CCAGCAACTCGGGCAAGAGC ACCATCGTCAAGCAGATGAA

```
                2120                2140                2160
                 *                   *                   *
                                 5'    1BF    3'
                                   GCCTGCAAG GAGTACAAGCCC
GATCATCCACAGCGGGGGCT TCAACCTGGACGCCTGCAAG GAGTACAAGCCCCTCATCAT
                    |||||||||||||||||| |||||
                    TTGGACCTGCGGACGTTC CTCAT
                  5'      mGzGR       3'

                2180                2200                2220
                 *                   *                   *
CTACAACGCCATCGACTCGC TGACCCGGATCATCCGGGCC CTGGCTGCCCTCAAGATCGA

                2240                2260                2280
                 *                   *                   *
TTTCCACAACCCTGACCGTG CCTACGACGCTGTGCAGCTC TTTGCTCTGACTGGCCCAGC

                2300                2320                2340
                 *                   *                   *
                  5'      4BF       3'
                      CCCGAGCTGCTGGGTGTC ATG
AGAGAGCAAGGGTGAGATTA CACCTGAGCTGCTGGGTGTC ATGCGACGGCTCTGGGCTGA

                2360                2380                2400
                 *                   *                   *
CCCAGGGGCCCAGGCCTGCT TTGGCCGCTCTAGCGAGTAC CACCTGGAGGACAATGCAGC

                2420                2440                2460
                 *                   *                   *
CTACTACCTGAACGACCTGG AGCGCATCGCAGCGCCCGAC TACATCCCCACGGTGGAGGA

                2480                2500                2520
                 *                   *                   *
TATCCTACGCTCCCGGGACA TGACCACGGGCATTGTGGAG AACAAGTTCACCTTCAAGGA

                2540                2560                2580
                 *                   *                   *
                                                      BamHI
GCTTACCTTCAAGATGGTGG ACGTGGGCGGGCAGAGGTCA GAACGCAAAA AGTGGATCCA

                2600                2620                2640
                 *                   *                   *
                                    SacI
TTGCTTTGAAGGCGTCACAG CCATCATCTTCTGTGTGGAG CTCAGTGGCTATGACCTGAA

                2660                2680                2700
                 *                   *                   *
GCTCTATGAGGACAACCAGA CGGTGAGTAGAGCCTAGGTT TCTACTGTTGGTTCCCAGGA

                2720                2740                2760
                 *                   *                   *
AGCAGGTGGACTTGTAGGAG CAGAGAGAGACTTTGGTCCA GGGCTGTTACTGAGCCTTGC

                2780                2800                2820
                 *                   *                   *
CCAGCCACGTGGACGGCGCT GTGAGTGGTTCCATAAGATG GGATGAGCACACCCCATGTT

                2840                2860                2880
                 *                   *                   *
TTATGTTGGGGGGTTGCAGG GGGGAATGTGGTGTGGAACA TGGTGAGTTCATGCATGACA
```

```
                2900                2920                2940
                 *                   *                   *
CGGGACTTCAGGGGCATTCT CGTCGAGTTATGAGCCCCAG TGGACAGGGTCATGATGGTA

                2960                2980                3000
                 *                   *                   *
TCCCATAAGACCTCAACTTT CTACGACTCCTTCAGCCTGA GGGACATCACTTTGACCTAG

                3020                3040                3060
                 *                   *                   *
ATGAACCTGCTCTGACACCT TCCAAATGGCACCACAACCC CTAGACTCCATAGGGCTCAG

                3080                3100                3120
                 *                   *                   *
CAGGCAAGGACCCCCCTCCT TTATCAGCTATGACGACGCC TCCCCCAACAGGTGGAACTT

                3140                3160                3180
                 *                   *                   *
GGGAACTTTCCTTATAGTGT TTGTTGGCTCCACTGCCCAG GTTTCCATGTCACCTTGCCC

                3200                3220                3240
                 *                   *                   *
TCAGTACAGTGAATACGTGA TTGTGCCTGTGCCACTTGCT GAGGAAATCCAACGTAAGGC

                3260                3280                3300
                 *                   *                   *
CTGGCTGGGCGTCACTGGTG GTCCATGTGGCAATGTAGCA AACCCACTAAGCTCCTACCA

                3320                3340                3360
                 *                   *                   *
GCACTGCAGGATATGTAGGT CCTGAGAACGGCAACCCCCG CCCAGGTCTCCTTCTTGCTC

                3380                3400                3420
                 *                   *                   *
TGAAGAGTCTAGCGATGATC TGGTTAAGAAGAGTAAGTCC AAGGTGCCAAGACCTGACCT

                3440                3460                3480
                 *                   *                   *
TGGGCTTGGTGGATAGGCTG TAGGGCCATTGTTGAGGTTC TTTGAGTCCCTTATCCTATG

                3500                3520                3540
                 *                   *                   *
CTTAAAACCAGTGTGTTGGA AGGGGTGTTAGGGGCATGAA ATAGGACATAGGCAACCTGA

                3560                3580                3600
                 *                   *                   *
AGGAGACCTAGGTCCAGTGG TTAGCTAGGAGATCTTGAGA GGGGGCTGCTGAAGAAACAG

                3620                3640                3660
                 *                   *                   *
TTAAGCAGAAGTAGGCCTTT TGCATGGAGAGGGTAGGCAG TATGCTCAGATCCGCCTGAG

                3680                3700                3720
                 *                   *                   *
GCAGAAGGCTAGACTTCCAA GCTACCTACACTCTTCCCAG GAACAGCTCTAGTAGAAGAG

                3740                3760                3780
                 *                   *                   *
TGGCAGTCAGACAAATGATA GTGGTCTGCTTGAGGGCTGT CTTCCGTAGTGGAGCTACTG
```

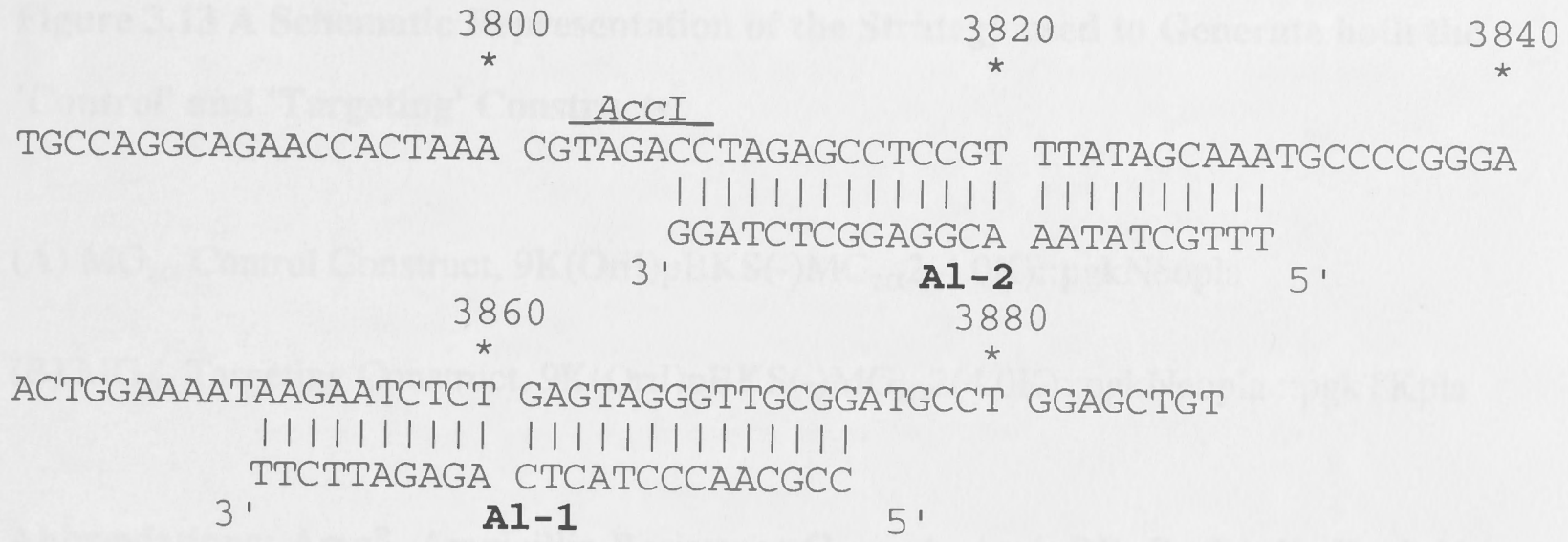

Table 3.2 The exon2/3 Boundary of the $HG_{z\alpha}$ and $MG_{z\alpha}$ Genes

(References: Padgett *et al.*, 1986; Matsuoka *et al.*, 1990)

Exon	5' Boundary	3' exon2/3 Boundary
Human Exon2	ttcctttccccag..AGTCGG	AACCAGACA **V** AGTCGGATG
Mouse Exon2	ttctctctttccttag..GTGAAG	AACCAGACG **V** AGCCGGATG
Consensus Sequence	ccccccccn...cag:G ttttttttt...cag:G	

Figure 3.13 A Schematic Representation of the Strategy used to Generate both the 'Control' and 'Targeting' Constructs.

(A) $MG_{z\alpha}$ Control Construct, 9K(OriI)pBKS(-)$MG_{z\alpha}$2(4.0K)::pgkNeopla

(B) $MG_{z\alpha}$ Targeting Construct, 9K(OriI)pBKS(-)$MG_{z\alpha}$2(4.0K)::pgkNeopla ::pgkTKpla

Abbreviations: Amp^R, Ampicillin Resistance Gene; A, *Acc*I; BB, *Bsa*BI; K, *Kpn*I; N, *Not*I; Neo^R, Neomycin Resistance Gene; prom, pgk, promoter; pla, polyadenylation signal; and TK, the Thymidine Kinase Gene.

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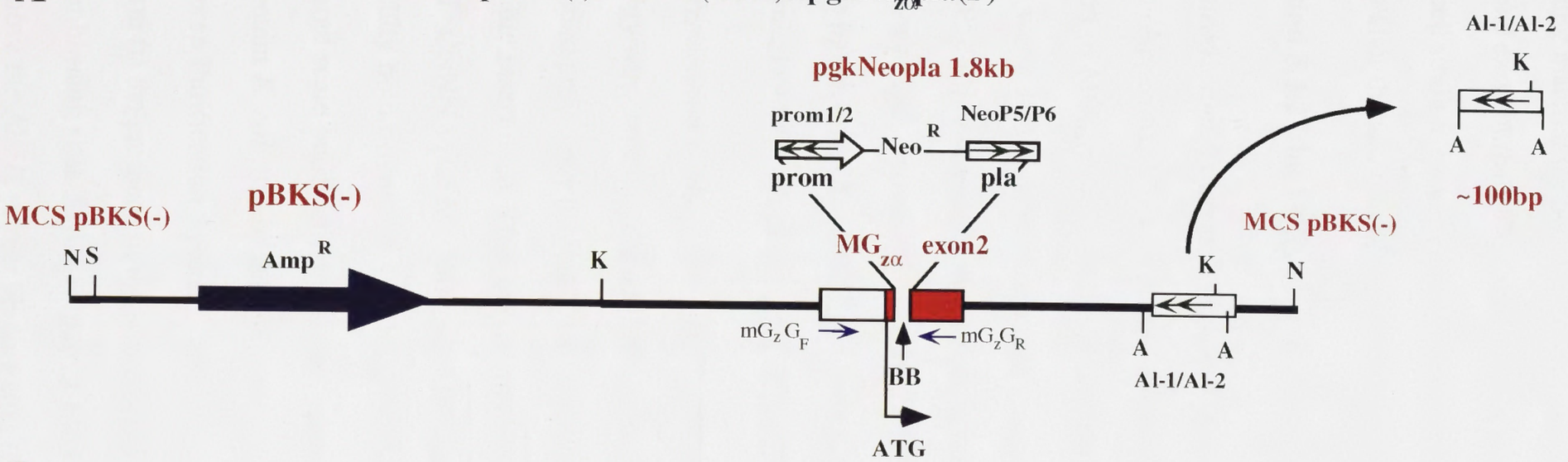
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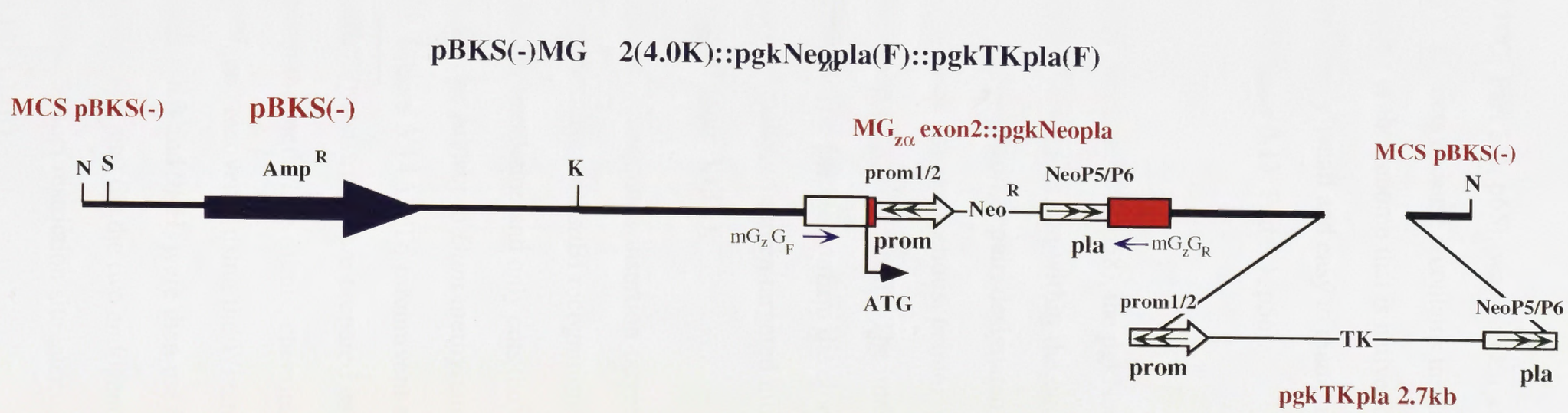
pgkNeopla 1.8kb
prom1/2
NeoP5/P6
Neo R
prom
pla
MCS pBKS(-)
pBKS(-)
Amp R
N S
K
MG zα
exon2
mG z G F
mG z G R
BB
ATG
K
N
A A
Al-1/Al-2
MCS pBKS(-)
Al-1/Al-2
K
A A
~100bp

B


pBKS(-)MG 2(4.0K)::pgkNeopla(F)::pgkTKpla(F)
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pBKS(-)
N S
Amp R
K
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prom1/2
NeoP5/P6
Neo R
mG z G F
prom
pla
mG z G R
ATG
MCS pBKS(-)
N
prom1/2
NeoP5/P6
TK
prom
pla
pgkTKpla 2.7kb

Part 3.3 Targeting the $MG_{z\alpha}$ Gene

Section 3.3.1 Introduction.

The pBKS(-)$MG_{z\alpha}$2(4.0K) construct (Figure 3.10C; Part 3.2, p65), was chosen as the starting material for both the control and targeting constructs because it contains the entire second exon of the $MG_{z\alpha}$ gene, within a large segment of the genome that is native to the ES cell strain to be targeted (C57BL/6), yet still, 'relatively' small and easy to manipulate, even after the addition of various selectable markers (Figure 3.13; Part 3.1, p56).

Section 3.3.2 The $MG_{z\alpha}$ Control Construct.

a) Introduction. To create the 'control' construct (PCR; Section 3.1.3, p58), the pgkNeopla gene (Appendix 2.3) was cloned into the lone *Bsa*B1 restriction site within the coding region of $MG_{z\alpha}$ exon2 (Figure 3.12; 3.14). The endogenous primer pair, designated Al-1/2, were also designed for use in combination with the exogenous primer pair (NeoP5/P6), to screening of homologous recombinants in targeted ES cells. The internal primers mG_zG_F and mG_zG_R (Table 2.1; Figure3.12)would be used to confirm the cloning of the pgkNeopla (Appendix 2.3) gene *in vitro* and eventually, to screen targeted mouse genomic DNA for the presence of the pgkNeopla gene (Figure 3.13A).

b) Preparation of the pBKS(-)$MG_{z\alpha}$2(4.0K) Subclone. A sequence insertion targeting strategy was chosen to disrupt $MG_{z\alpha}$ gene expression, because the *Bsa*BI recognition site identified previously (Figure 3.12), is close to the start of translation and only cuts the 4kb genomic insert once. This site, however, was found to be subject to *Dam* methylation* (GAT*CNNNATC; McClelland & Nelson, 1992; Figure 3.14A). To circumvent this difficulty both orientations of the pBKS(-)$MG_{z\alpha}$2(4.0K) construct were prepared using the large scale purification protocol, transformed into the methylase minus, chemically competent *E. coli* strain JM110 (Section 2.1.1, p30), and recovered using the Wizard® Miniprep Purification System. The clones, designated 8KB and 9KH, were then used to confirm (i) the presence of the unprotected *Bsa*BI restriction site; (ii) the two endogenous primer binding sites Al-1/2 (Figure 3.14C); and (iii) the 3' *Acc*I restriction site later used to remove the Al-1/2 primer binding sites (Figure 3.12; 3.14B).

Figure 3.14 Confirmation of pBKS(-)MG$_{z\alpha}$2(4.0K) *Bsa*BI Restriction Site and the Al-1/2 Primer Binding Sites.

(A) The DNA from the 9K pBKS(-)MG$_{z\alpha}$2(4.0K) plasmid was digested with either *Kpn*I, *Bsa*BI and/or *Bam*HI, and the fragments separated using agarose (1%) gel electrophoresis. Lane 1, uncut plasmid (9K); Lane 2, linearised plasmid (9K/*Hin*dIII); Lane 3, DNA from the 9K clone cut with fresh *Bsa*BI (9K/*Bsa*BI); Lane 4, 9K/*Bam*HI; Lane 5, 9K/*Bam*HI/*Bsa*BI; and Lane 6, 9K/*Kpn*I. This gel shows that 9K/*Bsa*BI (Lane 3) runs at the same level as uncut plasmid (Lane 1). The *Bsa*BI should also cut the characteristic MG$_{z\alpha}$2(2.0B) fragment (Part 3.2, p65), however, although *Bam*HI appears to cut out the predicted fragments, *Bsa*BI does not (Figure 3.10).

(B) The DNA from the methylase minus 8KB pBKS(-)MG$_{z\alpha}$(4.0K) clone, prepared earlier, was digested with either *Acc*I, *Kpn*I, *Bsa*BI and/or *Bam*HI and the fragments separated using agarose (1%) gel electrophoresis. Lane 1, uncut 8KB (control); Lane 2, linearised 8KB (8KB/*Hin*dIII); Lane 3, 8KB/*Bsa*BI; Lane 4, 8KB/*Bam*HI; Lane 5, 8KB/*Bam*HI/*Bsa*BI; Lane 6, 8KB/*Kpn*I; and Lane 7 8KB/*Acc*I. Points to note include the *Bsa*BI cut plasmid (Lane 3), the cleaved 2kb *Bam*HI fragment (0.47kb+1.53kb; Lane 5) and the 4kb *Acc*I DNA fragment (Lane 7) which coincides with the placement of the *Acc*I restriction site. In OriII (8K) the two *Acc*I restriction sites (the endogenous *Acc*I site and the MCS *Acc*I site) are at either end of the cloned 4kb insert.

(C) PCR Analysis of the Al-1/2 primer binding sites. These primers were used to amplify that region of the cloned MG$_{z\alpha}$2(4.0K) insert between themselves and the 4BF primer using PCR (1.5kb). Cycles 35x; ET 2min; T_E 72°C; AT 5sec; T_A 63°C. The product from each reaction was then run on a 1% agarose gel. The example shown is that of the Al-1 and 4BF applied to both orientations. Lanes 1 & 9, λ/*Hin*dIII (0.5µg); Lanes 2 & 8, ddH_2O (control); Lane 3, 250pg of clone 9K; Lane 4, 9K (25pg); Lane 5, 250pg of clone 8K; Lane 6, 8K (25pg); and Lane 7, 2500pg of C57BL/6 mouse genomic DNA. The product amplified from each clone also corresponds to the band amplified from the 'tail' DNA, indicating that both sets of primers are also native to the C57BL/6 mouse genome.

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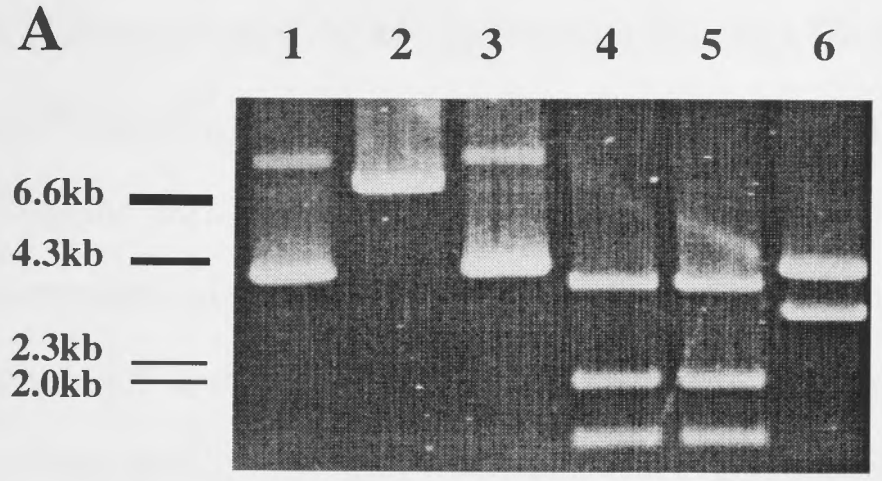
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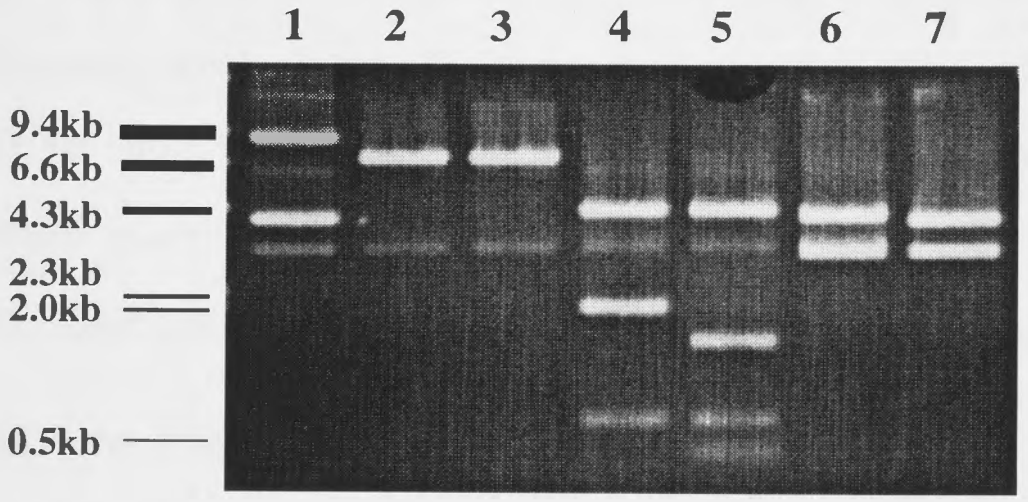
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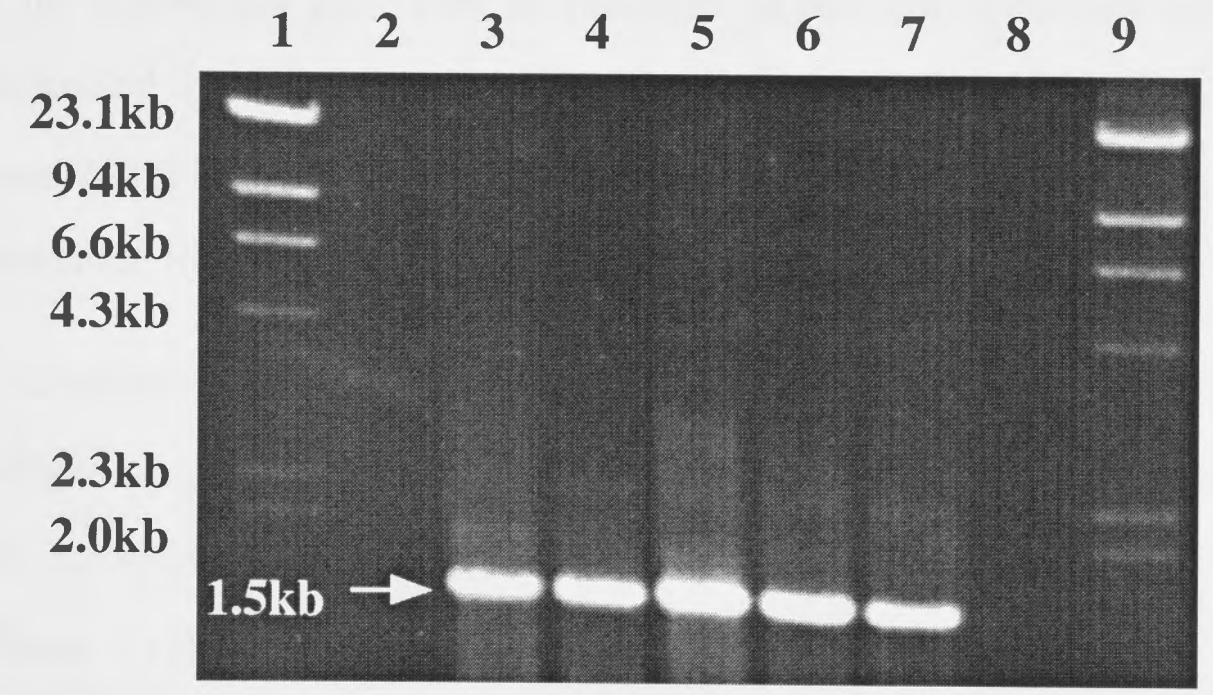
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c) Construction of the $MG_{z\alpha}$ Control Construct. To make the $MG_{z\alpha}$ control construct the pgkNeopla (1.8kb) gene was restricted from pBSK(+)pgkNeopla vector (Appendix 2.3) using the both *Hin*dIII and *Eco*RI, separated from the host vector using agarose (1%) gel electrophoresis and purified with the Gene Clean® Protocol (Section 2.3.3, p38). The over hanging 5' ends, of the pgkNeopla fragment, were then blunt ended (Section 2.3.4, p39), and the pBKS(-)$MG_{z\alpha}$2(4.0K) construct digested with *Bsa*BI (Section 2.1.2, p30). The blunt ends of the recipient vector were then dephosphorylated (Section 2.3.5, p39), and added to a ligation mix (Section 2.3.6, p40) containing the prepared pgkNeopla fragment. Following incubation the DNA was then transformed into chemically competent *E. coli* DH5α and prepared using the Wizard® Miniprep Purification System. The putative control constructs, pBKS() $MG_{z\alpha}$2(4.0K)::pgkNeopla, representing both orientations of the cloned pgkNeopla fragement, were designated 26b, 29b and 30b respectively.

d) Characterisation of the Putative Control Constructs. To confirm the presence of the pgkNeopla fragment and to identify its orientation, each putative control constructs was prepared using the large scale plasmid purification protocol, restricted (0.5μg) with either *Bam*HI and/or *Eco*RI and separated using agarose (1%) gel electrophoresis (Figure 3.15A). In this way it was found that clones 29b and 30b (Figure 3.16A), contain a copy of the pgkNeopla gene with its promoter in the same direction as the $MG_{z\alpha}$ gene, designated the forward orientation (F), pBKS(-)$MG_{z\alpha}$2(4.0K)::pgkNeopla(F), whilst clone 26b (Figure 3.16B) contains a copy of the cloned pgkNeopla insert in the reverse orientation (R), pBKS(-)$MG_{z\alpha}$2(4.0K)::pgkNeopla(R).

e) Confirmation of Orientation and Primer Binding Sites. PCR analysis with the Al-1 endogenous primer was used to determine whether the 'control' exogenous primer binding sites (prom1/2, NeoP5/P6; Figure 3.16), situated at either end of the pgkNeopla fragment (Figure 3.13), had survived blunt end cloning. This demonstrated that clones: 1a, 5b & 29b possess pgkNeopla(F); 21a, 26b & 51b possess pgkNeopla(R), whilst 31b does not contain the pgkNeopla fragment. Furthermore although 30b was previously thought to contain pgkNeopla(F) no band was amplified (Section 2.3.10, p42; Figure 3.15B), suggesting damage to the end of the pgkNeopla insert in this instance.

Figure 3.15 Characterisation of the Putative $MG_{z\alpha}$ Control Constructs.

(A) Diagnostic Restriction Analysis of the Putative Control Constructs. Lane 1, 26b/*Bam*HI; Lane 2, 26b/*Eco*RI; Lane 3, 29b/*Bam*HI; Lane 4, 29b/*Eco*RI; Lane 5, 30b/*Bam*HI; Lane 6, 30b/*Eco*RI; Lane 7, 9KH/*Bam*HI; and Lane 8, 9KH/*Eco*RI. Note that the size of each linearised clone (8.7kb; Lanes 2, 4 & 6), and the small *Bam*HI fragment (1.3kb; Lanes 1, 3, 5 & 7), remain the same, no matter what the orientation of the pgkNeopla insert, whilst the $MG_{z\alpha}2$(2.0B) genomic fragment, characteristic of the $MG_{z\alpha}2$(4.0K) cloned insert is lost. In the forward orientation the two new *Bam*HI fragments will be 2.8kb and 1.1kb in size. In the reverse orientation the *Bam*HI restriction site, within the pgkNeopla gene, will have moved to the opposite end of the insert, so the predicted fragments will then be 1.8 and 1.9kb in size (the 'doublet'; Lane 3; Figure 3.16).

(B) The PCR protocol was conducted using 250ng of template DNA and the primer pairs pgkprom1/Al-1 (R; Lanes 2 to 14) and NeoP5/Al-1 (F; Lanes 16 to 28). Cycles 35x; ET 2min; T_E 72°C; AT 5sec; T_A 63°C. The PCR product (1.8kb) was then run on a 1% agarose gel. Lanes 1 & 15, λ/*Hin*dIII (0.5µg); Lanes 2 & 16, ddH_20; Lanes 3 & 17, clone 9KH (negative control 1); Lanes 4 & 18, 1a; Lanes 5 & 19, 21a; Lanes 6 & 20, 5b; Lanes 7 & 21, 26b; Lanes 8 & 22, 29b; Lanes 9 & 23, 30b; Lanes 10 & 24, 31b; Lanes 11 & 25, 51b; Lanes 12 & 26, mouse genomic C57BL/6 DNA; Lane 13, 26b (R, positive control); Lane 27, 29b (F, positive control); and Lanes 14 & 28, ddH_2O.

(C) An example of 'nested' PCR optimised using the control construct 29b. The first round of amplification uses the outside primers, Al-1/NeoP5 [Cycles, 20x; Extension Time (ET), 2min; Extension Temperature (T_E), 72°C; Annealing Time (AT), 5 sec & Annealing Temperature (T_A), 67°C], and the second the two inside primers, Al-2/NeoP6 (Cycles, 35x; ET, 2min; T_E, 72°C; AT, 5sec & T_A, 65°C). The final PCR product (1.8kb) was then run on an agarose (1%) gel. Lane 1, l/HindIII; Lane 2, 10fg; Lane 3, 100fg; Lane 4, 1pg; Lane 5, 10pg; and Lane 6, 100pg. The size marker, λ/*Hin*dIII is reproduced on the left. To minimise cross contamination the template was added to each reaction mix at an increasing concentration. Gel courtesy of Dr Klaus Matthaei.

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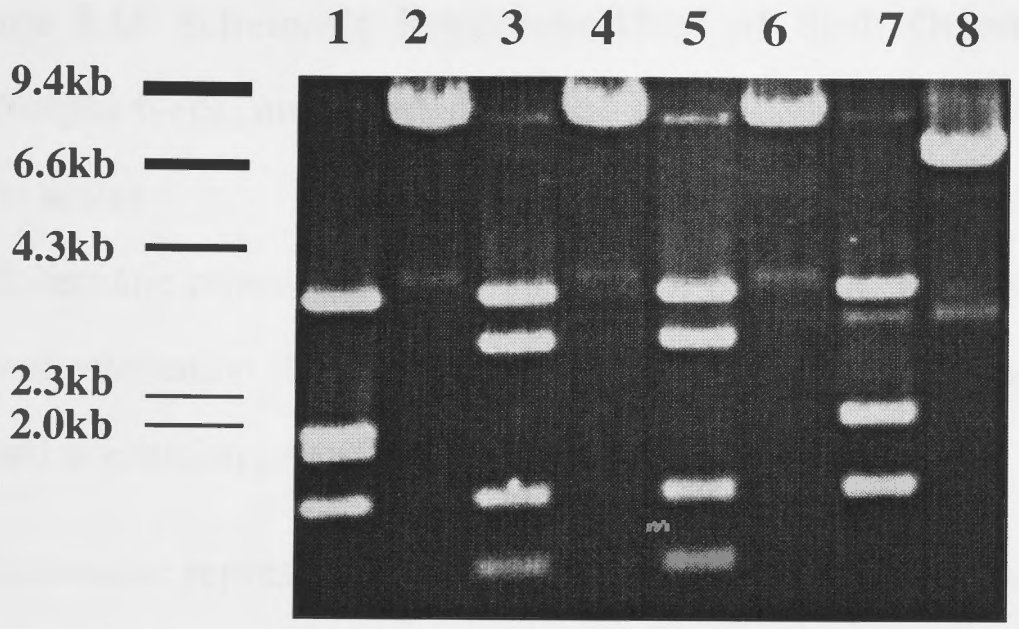
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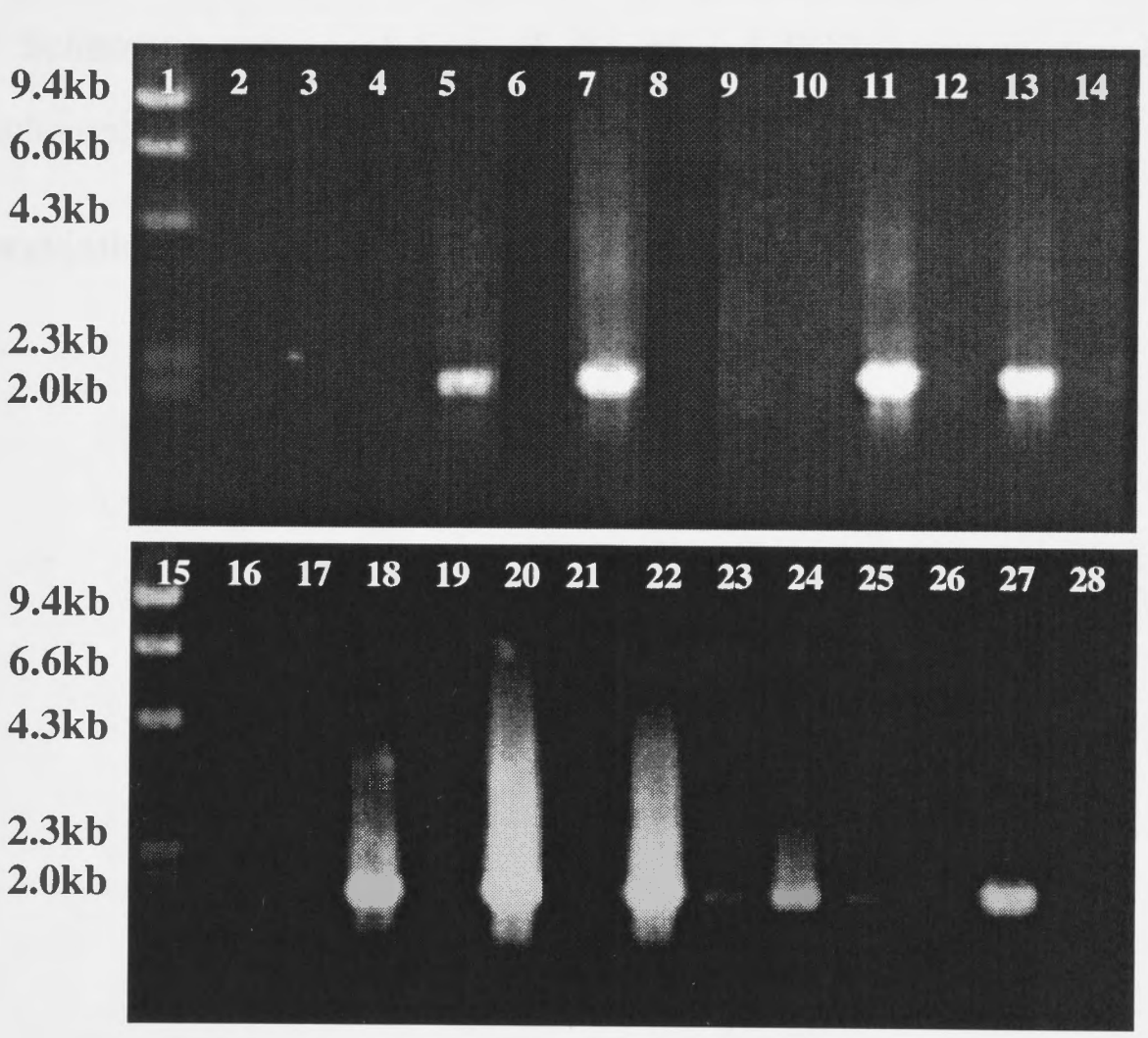
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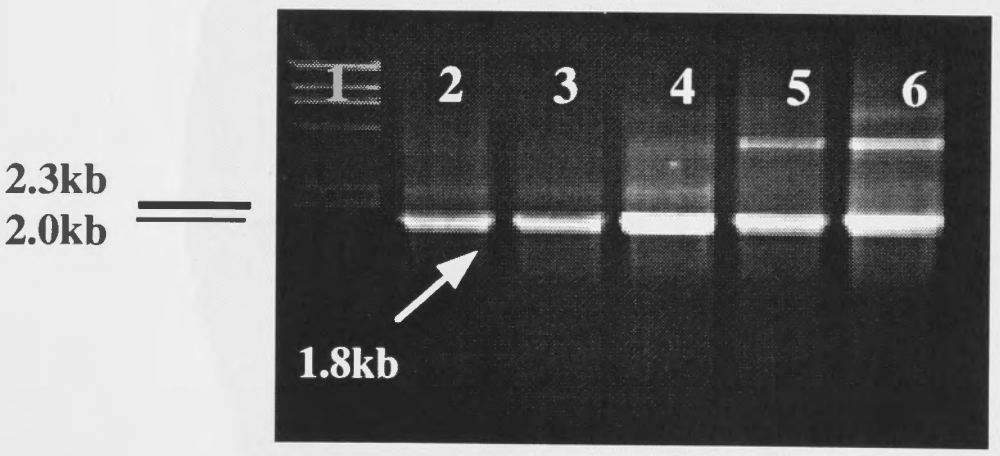
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Figure 3.16 Schematic Representations of Both Orientations of the Cloned pgkNeopla Gene, and 'Nested' PCR.

(not to scale)

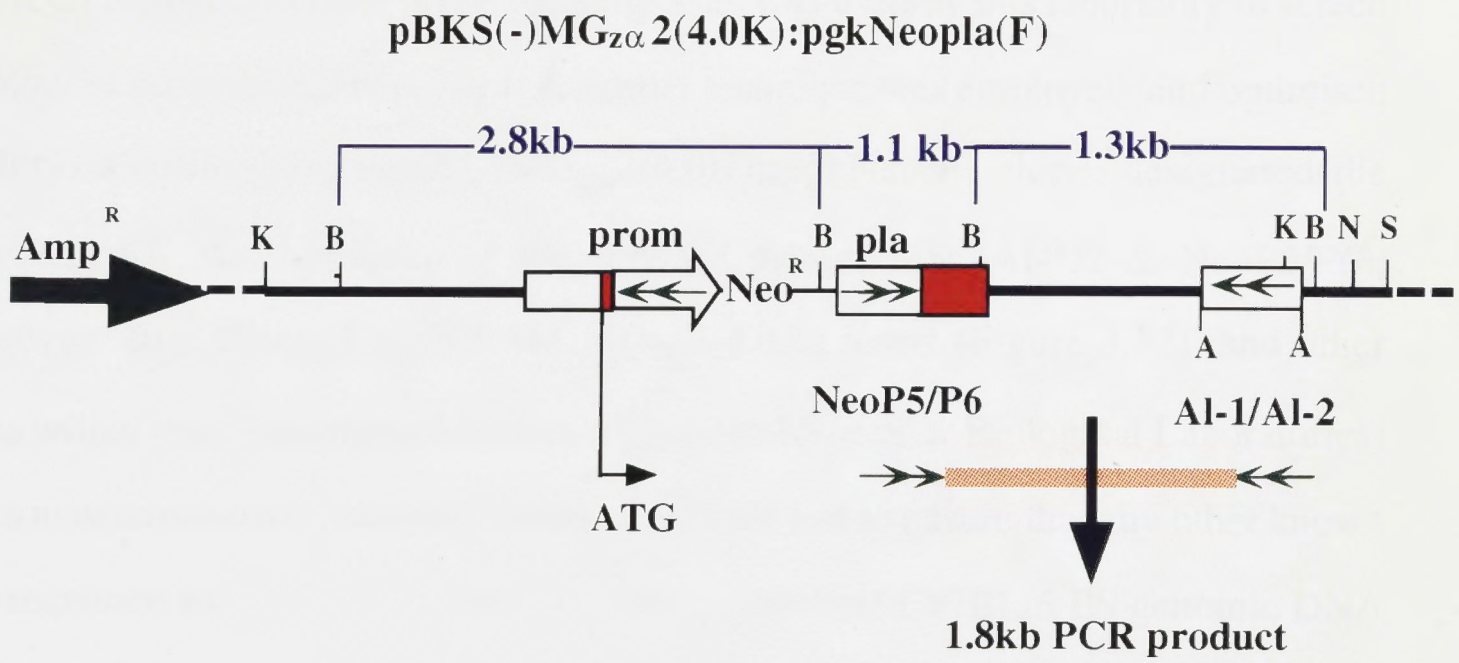
(A) Schematic representation of the control construct, with the pgkNeopla gene in the forward orientation (F), showing restriction, and primer binding sites for the two sets of forward orientation primer sets; NeoP5/P6 and Al-1/2.

(B) Schematic representation of the control construct, with the pgkNeopla gene in the reverse orientation (R), showing restriction, and primer binding sites for the two sets of reverse orientation primers prom1/2 and Al-1/2.

(C) Schematic representation of the nested PCR strategy developd to identify homologous recombination in ES cells *in vitro*.

Abbreviations: A, *Acc*I; B, *Bam*HI; N, *Not*I; S, *Sac*I; K, *Kpn*I

A Clones 29b & 30b



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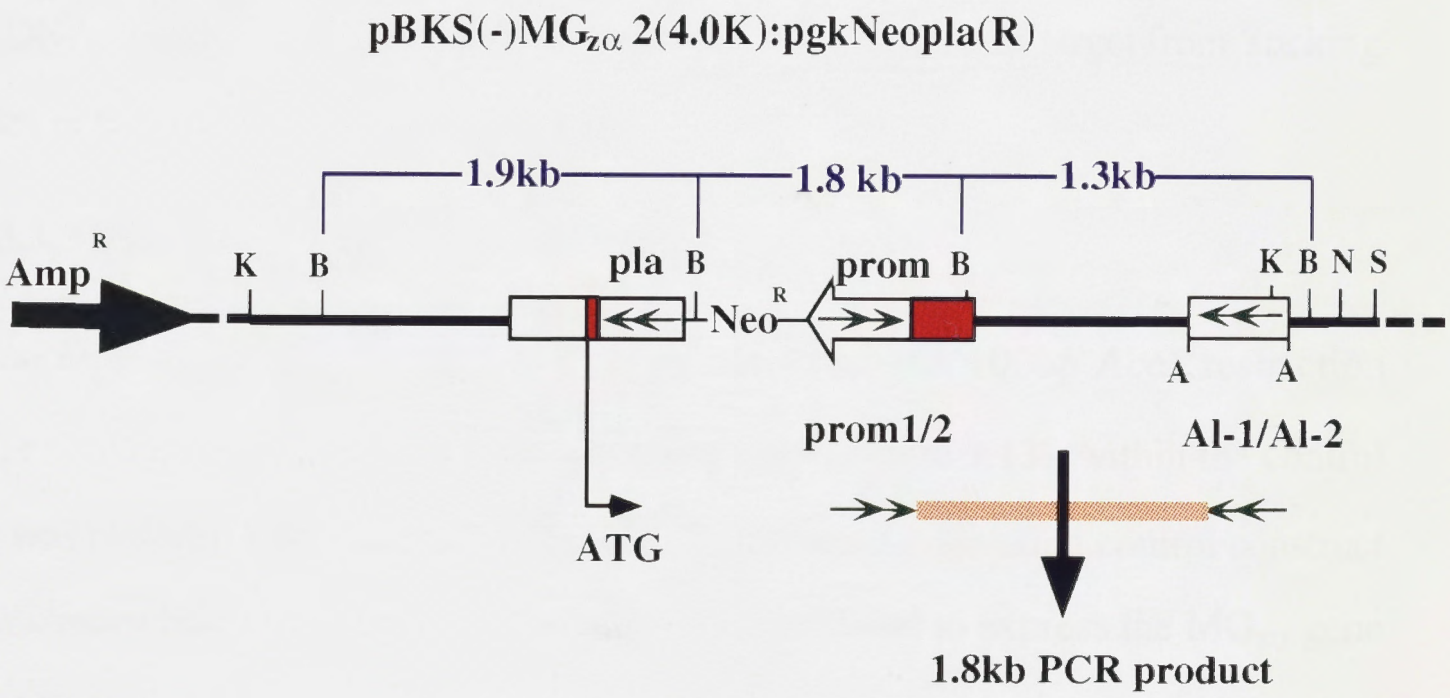

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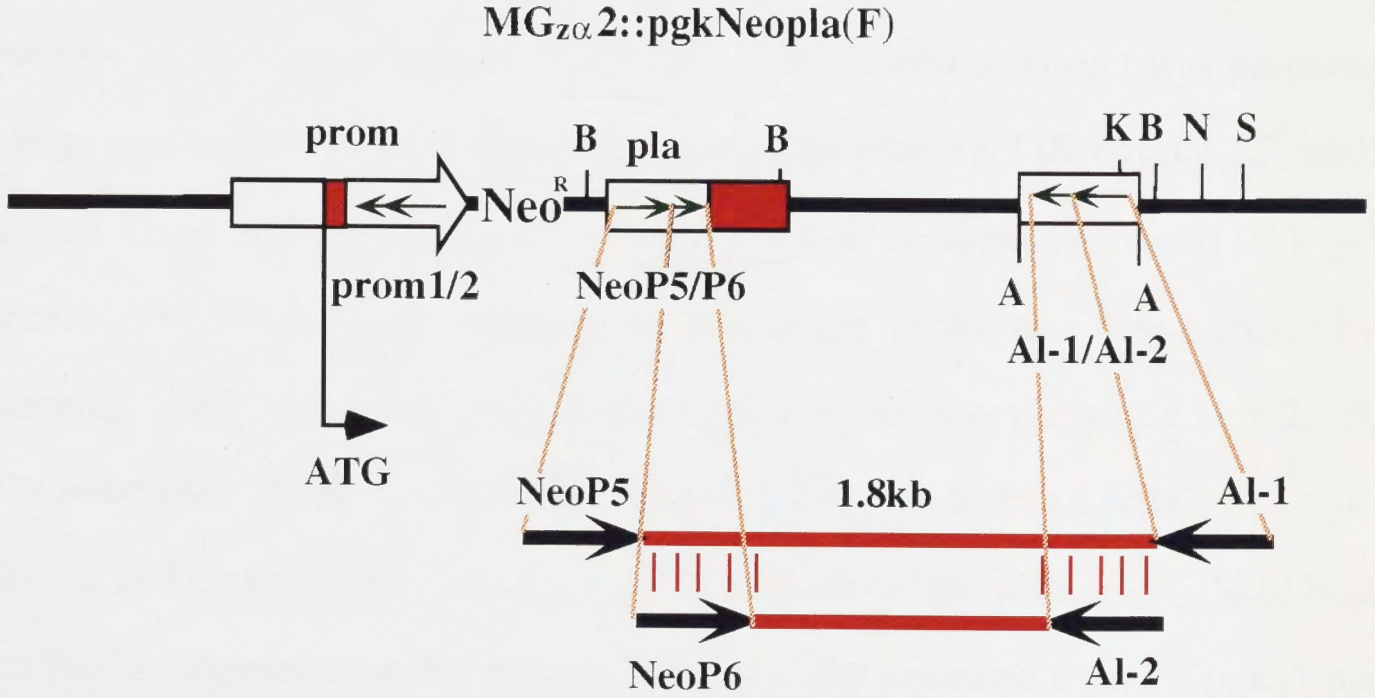

f) Optimising the 'Nested PCR' Strategy. A 'nested PCR' protocol (Nitschke *et al.*, 1993; Figure 3.16C) using the control primer binding sites was used by this laboratory to screen for homologous recombination *in vitro*. A similar technique was employed, and optimised using dilutions of the 29b, pBKS(-)$MG_{z\alpha}$2(4.0K)::pgkNeo(F) clone, designated the control construct. The sequence of the forward primer sets (Al-1/2 & NeoP5/P6), however, were first checked against the $MG_{z\alpha}$2(4.0K) insert (Figure 3.12), and other sequences within the Genbank and EMBL (European Molecular Biological Laboratories) data bases to determine the specificity of these primers and to ensure that any other known genomic sequence will not corrupt the screening of targeted C57BL/6 ES genomic DNA for the pgkNeopla gene (Part 3.1, p56). The control template DNA was prepared using the Wizard® Miniprep Purification System Protocol and diluted in 100pg/μL of mouse genomic DNA, which acts as a carrier, to prevent the small amount of target from 'sticking' to the sides of the reaction tubes (Figure 3.15C).

Section 3.3.3 The $MG_{z\alpha}$ Targeting Construct.

a) Introduction. To create the $MG_{z\alpha}$ targeting construct the 100bp *Acc*I restriction fragment, encompassing the Al-1/2 primer binding sites (Figure 3.13), within the control construct was replaced with the pgkTKpla gene. The forward orientation control construct (29b) was chosen because, the transcriptional machinary used to express the $MG_{z\alpha}$ gene should not hinder the expression of the Neo^r gene following its specific integration into the targeted ES cell genome (Capecchi, 1989).

b) Construction of the $MG_{z\alpha}$ Targeting Construct. The control construct was prepared using the large scale purification protocol, digested (2μg) with *Acc*I (Section 2.1.2, p30) and separated from the Al-1/2 primer binding sites through agarose (1%) gel electrophoresis. The DNA band containing the rest of the vector was then purified by freeze squeezing, blunt ended (Section 2.3.4, p39) and dephosphorylated (Section 2.3.5, p39). At the same time pBSK(+)pgkTKpla (Appendix 2.4) was digested (2μg), first with *Hin*dIII/*Eco*RI, and then with *Pvu*I (Section 2.1.2, p30), to cut the pBSK(+) (2.9kb) host vector, into smaller fragments, so that it could be more easily separated from the pgkTKpla gene (2.7kb) using agarose (1%) gel electrophoresis (Figure 3.17A). The pgkTKpla

Figure 3.17 Cloning the pgkTKpla gene.

(A) Purification of the pgkTKpla gene fragment. Lane 1, The top band contains the pgkTKpla gene, whilst the lower bands represent the remainder of the pBSK(+) cloning vector following digestion with *Pvu*I. The molecular weight standard, λ/*Acc*I (Lane 2).

(B) Restriction mapping the putative targeting constructs. Lane 1, Clone 12a digested with *Hin*dIII (12/*Hin*dIII); Lane 2, 57a/*Hin*dIII; Lane 3, 80a/*Hin*dIII; Lane 4, pBSK(+)pgkTKpla/*Hin*dIII; Lane 5, 29b/*Hin*dIII; Lane 6, 12a/E*co*RV; Lane 7, 57a/*Eco*RV; Lane 8, 80a/*Eco*RV; Lane 9, pBSK(+)pgkTKpla/*Eco*RV; Lane 10, 29b/*Eco*RV; Lane 11, 12a/*Bam*HI; Lane 12, 57a/*Bam*HI; Lane 13, 80a/*Bam*HI; Lane 14, pBSK(+)pgkTKpla/*Bam*HI; Lane 15, 29b/*Bam*HI; Lane 16, λ/*Acc*I (0.5μg); and Lane 17, λ/*Hin*dIII (0.5μg). Note the size change of the 1.3kb *Bam*HI fragment present in the control construct (Lane 15), and encompassing the 100bp *Acc*I fragment to 4kb in the targeting construct (Lanes 11, 12 & 13), due to the addition of the 2.7kb pgkTKpla fragment. The 1.6kb (Lane 6) and 2.1kb (Lanes 7 & 8) *Eco*RV fragments are indicative of the pgkTKpla(R) and pgkTKpla(F) orientations respectively (Figure 3.18).

(C) PCR analysis of clone 57, pBKS(-)$MG_{z\alpha}$2(4.0K)::pgkNeopla(F)::pgkNeopla(F), using the primers NeoP5 (pgkNeopla) and prom1 (pgkTKpla). Cycles, 35x; ET, 2min; T_E, 72°C; AT, 5sec & T_A, 65°C. Following the reaction PCR product (1.9kb) was run on a agarose (1%) gel. Lane 1, λ/HindIII (0.5μg); Lane 2, ddH_2O; Lane 3, 2.5pg; Lane 4, 25pg; Lane 5, 250pg; and Lane 6, 2500pg. Note that to minimise cross contamination the template was added from the lowest to the highest dilution.

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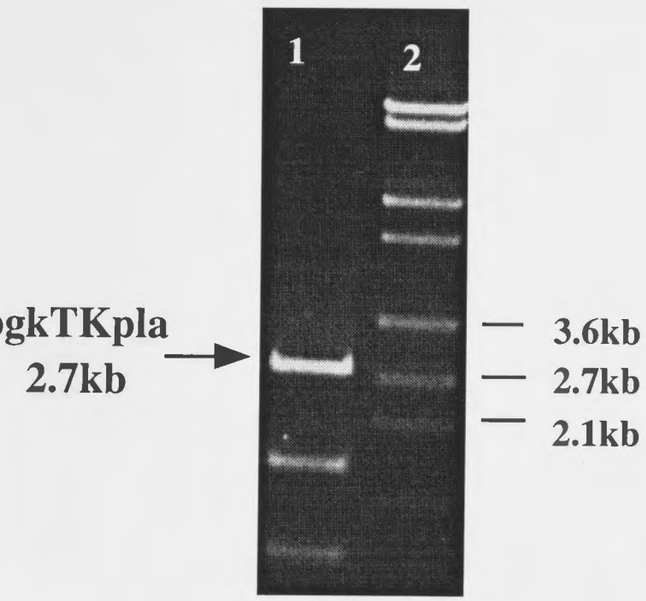
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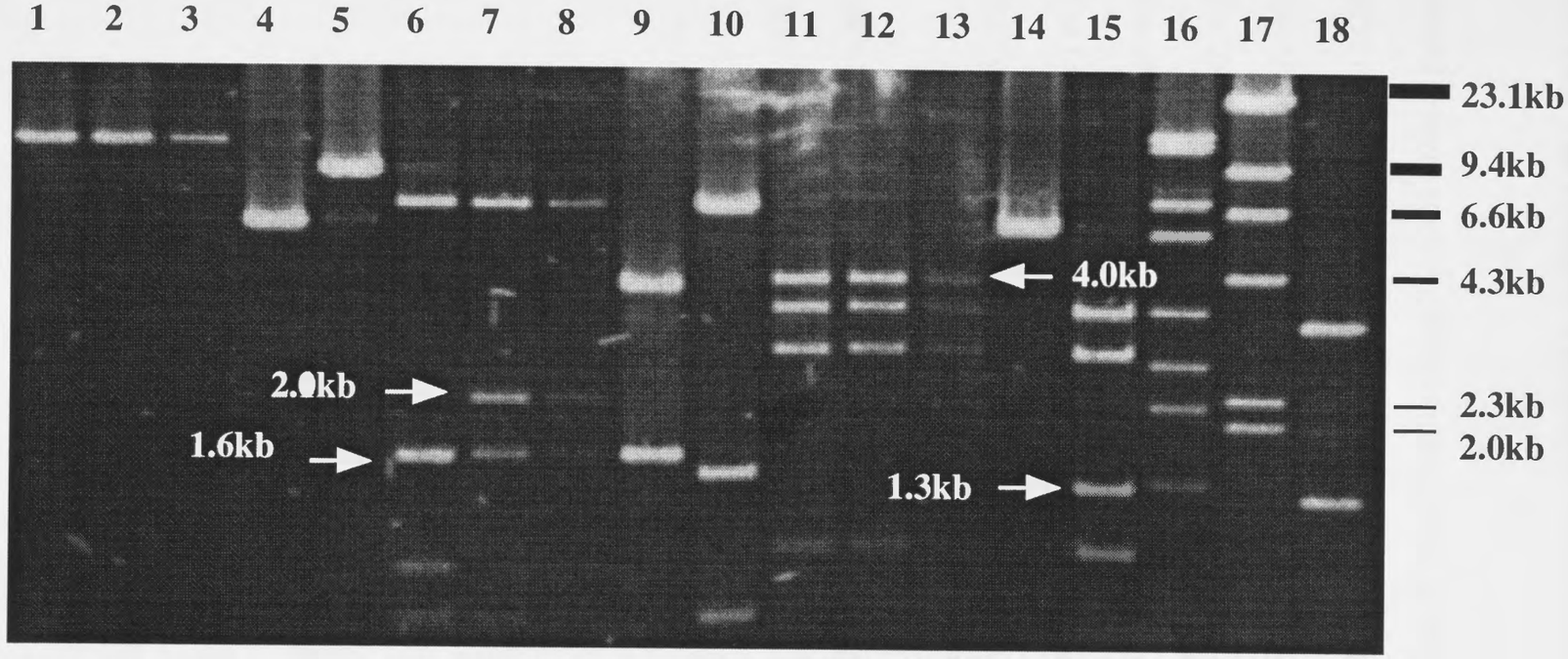
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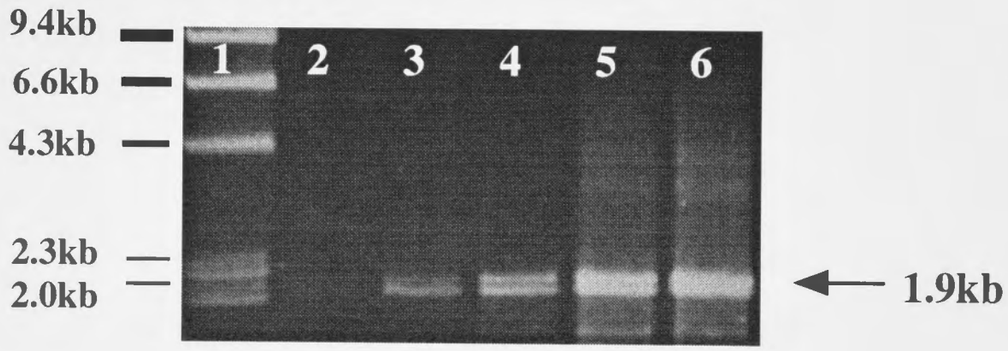
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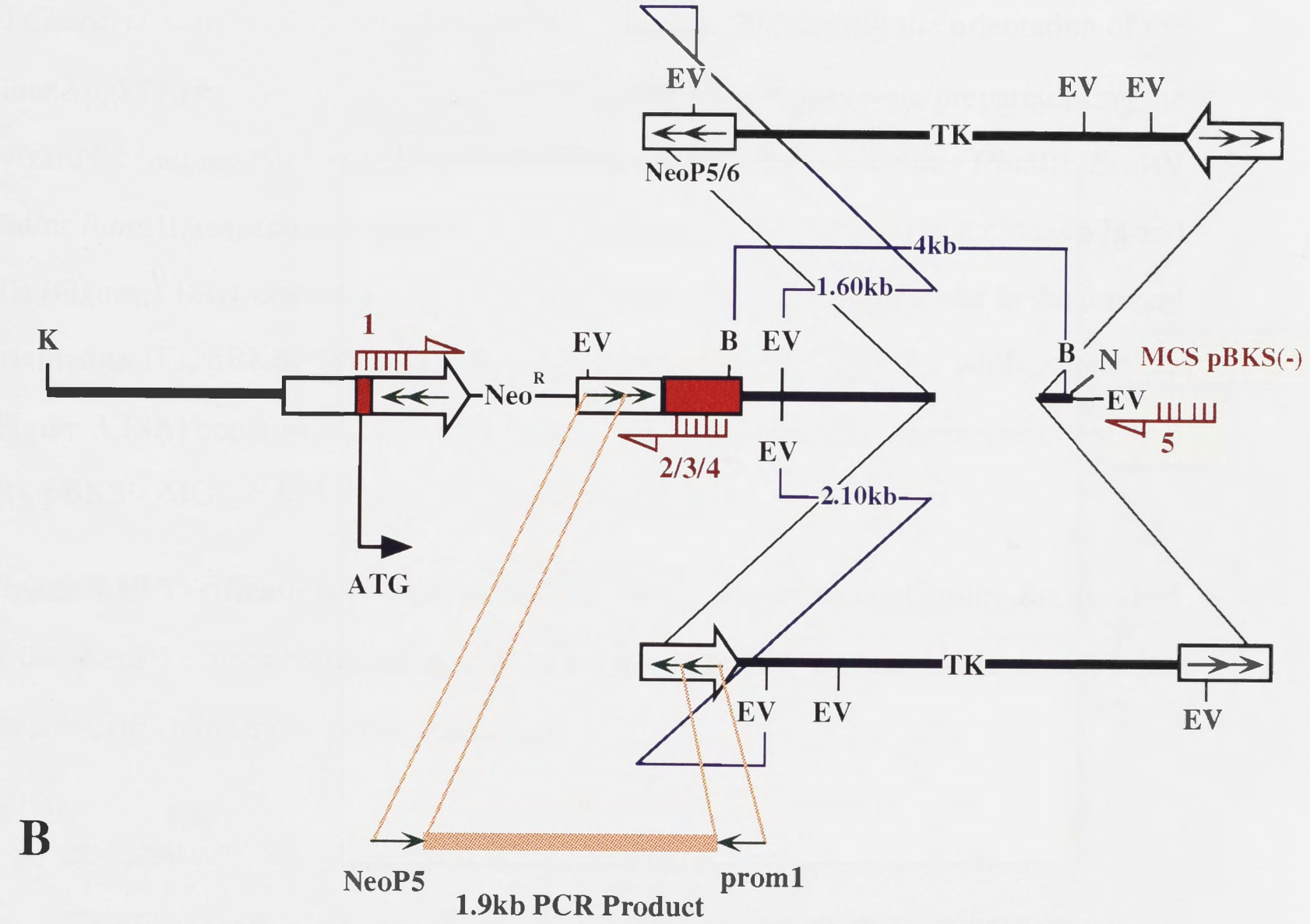
Figure 3.18 The Putative Targeting Constructs.

Schematic representation of both orientations of the cloned pgkTKpla gene, present within the targeting constructs. Predicted *Eco*RV fragment sizes for pgkTKpla(F): 6.50kb; 2.10kb; 1.50kb; 0.60kb; 0.17kb; & 0.26kb and for pgkTKpla(R): 6.50kb; 1.60kb; 0.62kb; 0.17kb; 1.50kb & 0.26kb (Figure 3.17B). Also shown are the sequencing primers used to verify each targeting construct. They include, anti-1BF(2), anti-4BF(3); $mG_zG_F(1)$, $mG_zG_R(4)$ used to sequence into the pgkNeopla fragment, and the universal primer to obtain sequence from the end of the pgkTKpla gene.

Abbreviations: B, *Bam*HI; EV, *Eco*RV K, *Kpn*I; N, *Not*I; Neo^R, the neomycin resistance gene; and TK, the thymidine kinase gene.

A **Clone 12**

pBKS(-)MG $_{z\alpha}$ 2(4.0K)::pgkNeopla(F)::pgkTKpla(R)



Clones 57 & 80

pBKS(-)MG $_{z\alpha}$ 2(4.0K)::pgkNeopla(F)::pgkTKpla(F)

fragment was then blunt ended, added to a ligation mix containing the prepared control construct (see above), incubated and the DNA transformed into chemically competent *E. coli* XL1-Blue cells (Section 2.1.1, p30). The presence of the cloned fragment was first identified using colony cracking, and later confirmed through the use of restriction analysis. The putative targeting construct pBKS()$MG_{z\alpha}$2(4.0K)::pgkNeo(F)::pgkTKpla containing clones were designated 12a, 57a and 80a respectively.

c) Characterisation of the $MG_{z\alpha}$ Targeting Construct. To identify the orientation of the cloned pgkTKpla gene, each of the putative targeting constructs were prepared using the Wizard® Miniprep Purification System, and digested (250ng) with either *Hin*dIII, *Eco*RV and/or *Bam*HI, respectively (Figure 3.17B). In this way it was found that clones 57a and 80a (Figure 3.18B), contain a copy of the pgkTKpla gene with its promoter in the forward orientation (F), pBKS(-)$MG_{z\alpha}$2(4.0K)::pgkNeopla(F)::pgkTKpla(F), whilst clone 12a (Figure 3.18A) contains a copy of the cloned pgkTKpla insert in the reverse orientation (R), pBKS(-)$MG_{z\alpha}$2(4.0K)::pgkNeopla(F)::pgkTKpla(R).

Figure 3.19 Verification of Primer Binding Sites. Sequence confirming the survival of the NeoP5 primer binding site, critical for the nested PCR screening protocol, using the anti-1BF endogenous primer binding site.

```
3'      1BF                5'
  CCCGAACATGAGGAACGTCCG
  ||||||||||||||||||||
  GGGCTTGTACTCCTTGCAGGC GTCCAGGTTG AAGCCCCCGC TGTGGATGAT
5'   anti-1BF           3'

  |----> pgkNeopla
CTAGCTTCTG ATGGAATTAG AACTTGGGAA AAACAATACT GAGAATGAAG

                  3'        NeoP5              5'
                    CCGACGACTAGAGCAAGAAGTCCG
                    ||||||||||||||||||||||||
TGTATGTGGA ACAGAGGCTGCTGATCTCGTTCTTCAGGC
```

d) Verification of the Targeting Construct. As a final measure of quality and to ensure that no damage had been done to the ends of either the pgkTKpla or pgkNeopla selection marker during blunt end cloning, the DNA from each putative targeting construct was sequenced (Figure 3.19), and subjected to PCR analysis (Figure 3.17C; 3.18).

Section 3.3.4 *In Vivo* Targeting the $MG_{z\alpha}$ Gene.

a) Preparation and Electroporation of the $MG_{z\alpha}$ Targeting Construct. Clone 57a pBKS(-) $MG_{z\alpha}$2(4.0K)::pgkNeopla(F)::pgkTKpla(F) was chosen as the targeting construct, primarily because the direction of the pgkTKpla promoter does not oppose the expression of either the $MG_{z\alpha}$ gene or the pgkNeopla genes (Section 3.3.3, p91). Prior to electroporation into ES cells this construct was first prepared using the Qiagen Midiprep® Purification System and cut with *Not*I to linearise the clone, allowing it to effectively integrate into the genome. The DNA was then electroporated into C57Bl/7 ES cells.

b) Isolation of an ES cell line which is Heterozygous for $MG_{z\alpha}$. The transformed cells were grown on media containing both neomycin and gancyclovir (Part 3.1, p56), and the surviving clones (up to 300) screened using the nested PCR protocol developed previously. Putative homologous recombinants were then grown in culture and subjected to Southern analysis for final confirmation that these clones did possess a specifically integrated copy of the pgkNeopla gene (Figure 3.20): The genomic DNA from seven of these ES cell clones was prepared, digested (5µg) with *Kpn*I, and *Hin*dIII/*Eco*RI. The DNA fragments from each sample were then separated by agarose (1%) gel electrophoresis, transferred to PCNM using alkaline transfer and subjected to high stringency hybridisation with an exon2 specific radiolabelled probe, representing the region between the mG_zG_F and mG_zG_R primer binding sites (Figure 3.12; 3.13; 3.20). The confirmed homologous recombinants were grown in culture, directly injected into BALB/c mouse blastocysts and implanted into pseudopregnant female foster mothers. This has resulted in the growth of a number of chimæras (Figure 3.21), which are currently being bred to determine germline transmission of the targeted allele.

Special Acknowledgments. PCR and Southern screening of the large numbers of homologous recombinants, blastocyst injection and the supervision of mouse breeding was conduct by and would not have been possible without the extensive technical expertise possessed by the Gene Targeting Group at the John Curtin School of Medical Research, the Australian National University. In particular Dr Klaus Matthaei and Ginny Sargent for their patience.

Figure 3.20 Southern Analysis of Putative ES Cell Recombinants.

PCR probe analysis of the genomic DNA prepared from the putative ES homologous recombinants (one to seven). Lane 1, Clone 1/*Kpn*I; Lane 2, Clone1/*Hin*dIII/*Eco*RI; Lane 3, Clone 2/*Kpn*I; Lane 4, Clone 2/*Hin*dIII/*Eco*RI; Lane 5, Clone 3/*Kpn*I; Lane 6, Clone 3/*Hin*dIII/*Eco*RI; Lane 7, Clone 4/ *Kpn*I; Lane 8, Clone 4/*Hin*dIII/*Eco*RI; Lane 9, Clone 5/*Kpn*I; Lane 10, Clone 5/*Hin*dIII/*Eco*RI; Lane 11, Clone 6/*Kpn*I, Lane 12, Clone 6/*Hin*dIII/*Eco*RI; Lane 13, Clone 7/*Kpn*I; and Lane 14, Clone 7/*Hin*dIII/*Eco*RI. The $MG_{z\alpha}2$(4.0K) genomic fragment, isolated to create the targeting and control constructs is endogenous to the ES cell genome, therefore, if specific integration has occurred in at least one allele of the gene, then this fragment will increase in size, to 5.8kb because of the presence of the pgkNeopla selection marker in that allele (1.8kb; Lanes 3, 5, 7, 9 & 11). Furthermore there is also a change in size of the original $MG_{z\alpha}2$(7.0H/E) from 7kb to 8.8kb in size (Lanes 2, 4, 6, 8 & 10) for the same reason. Note also the 4kb and 7kb fragments present in each of these lanes represents the other, non targeted allele of the gene region. Both gene alleles in Clone 1 appear to have escaped targeting (Lanes 1 & 2), whilst Clone 7 appears to have suffered multiple insertions of the targeting construct (Lanes 13 & 14).

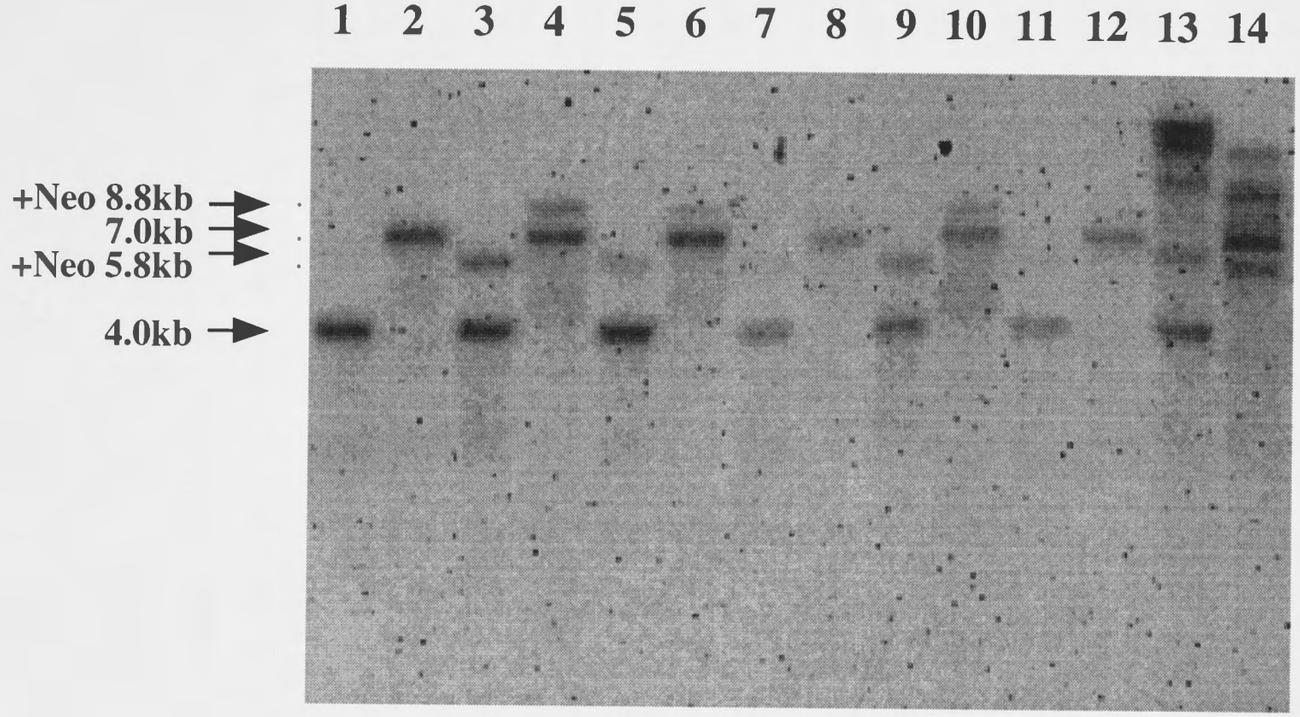

1 2 3 4 5 6 7 8 9 10 11 12 13 14
+Neo 8.8kb
7.0kb
+Neo 5.8kb
4.0kb

Figure 3.21 The $MG_{z\alpha}$ Knockout Chimæra

Shown is a photograph taken of one of the chimæric animals which have recently been produced by the Gene Targeting Facility. Note the black/white coat colouration, derived from the endogenous white BALB/c ES cells and the introduced black C57BL/6 ES cell line. Also present are litter mates and mother.

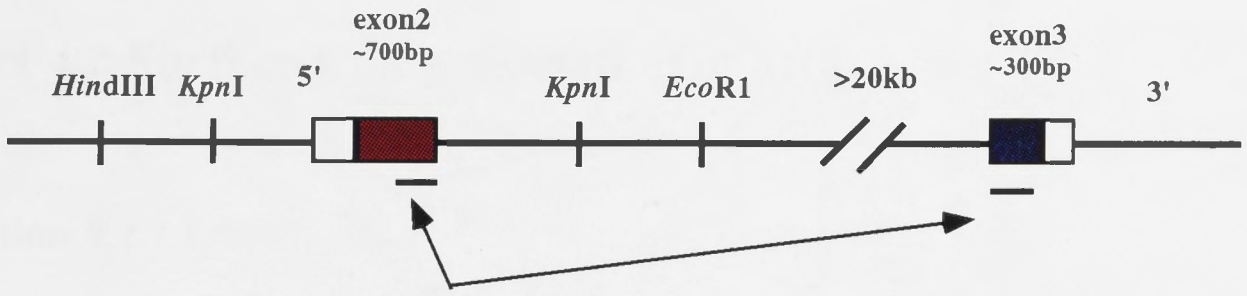
Chapter 4 Attempts to Clone and Characterise a Full-length $MG_{z\alpha}$ cDNA Clone.

Part 4.1 Introduction.

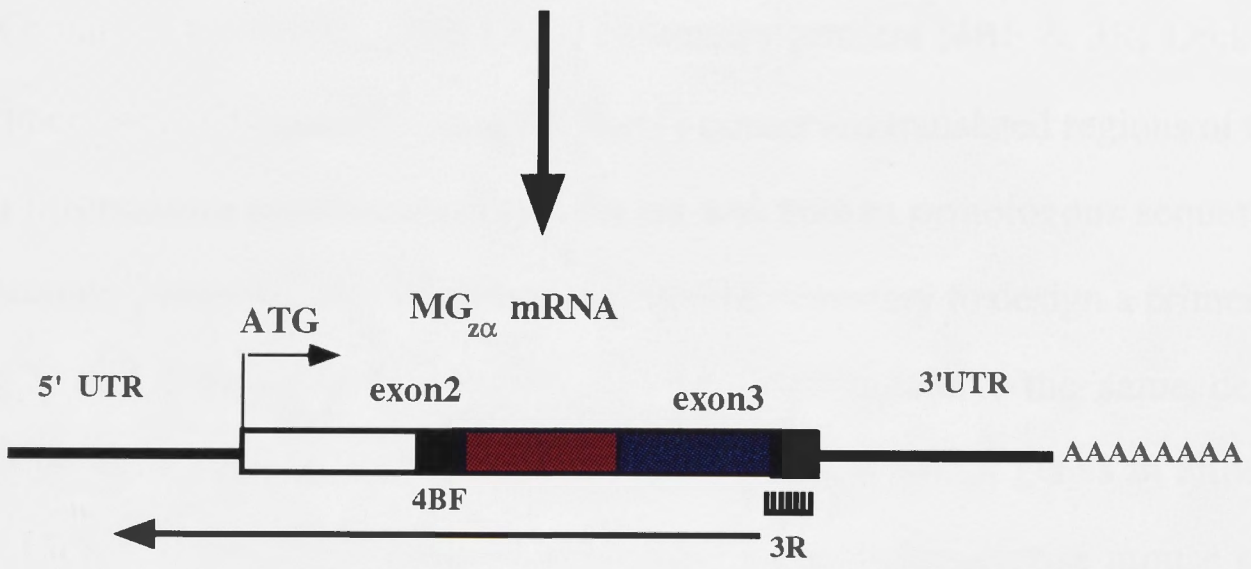
The major challenge posed by gene targeting is to prove that the phenotype of the knockout animal is directly due to the disruption of the gene targeted. This is especially difficult if there is no obvious phenotype, or if the expressed phenotype is due to a secondary interaction with some other protein (Chapter 5, p109). A common approach, although not the only one, is to generate a full length cDNA clone of the functional gene to see if this will rescue the phenotype of the knockout animal. A full length cDNA, in association with an *in vitro* expression system, can also be used to produce large amounts of the gene product. This chapter details the steps taken to generate a cDNA copy of the $MG_{z\alpha}$ mRNA. Time constraints and the lack of appropriate material meant that the construction and screening of a C57BL/6 (mouse brain) cDNA library was not initially considered, instead a reverse transcriptase-polymerase chain reaction (RT-PCR) strategy similar to that which was used to obtain the partial $MG_{z\alpha}$ cDNA clone was attempted (Leck, 1993; Figure 4.1).

Figure 4.1 The Cloning of a Partial $MG_{z\alpha}$ cDNA Fragment.

Both the translated (exon2, red; exon3, blue) and untranslated regions of each exon are shown (top). Note that the position and size of exon3 is assumed from the orthologous rat and human $G_{z\alpha}$ gene regions. The first strand synthesis is primed from the 3' oligonucleotide (3R) and amplified using both the 3R and 4BF in a single reaction mix (from Leck, 1993; see also Figure 3.5B).

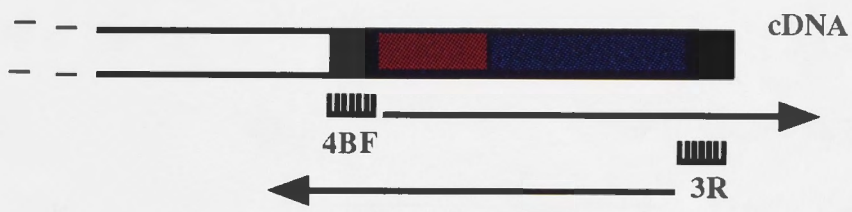


Regions of the Genome Corresponding to the Partial $MG_{z\alpha}$ cDNA Clone



First Strand Synthesis of cDNA

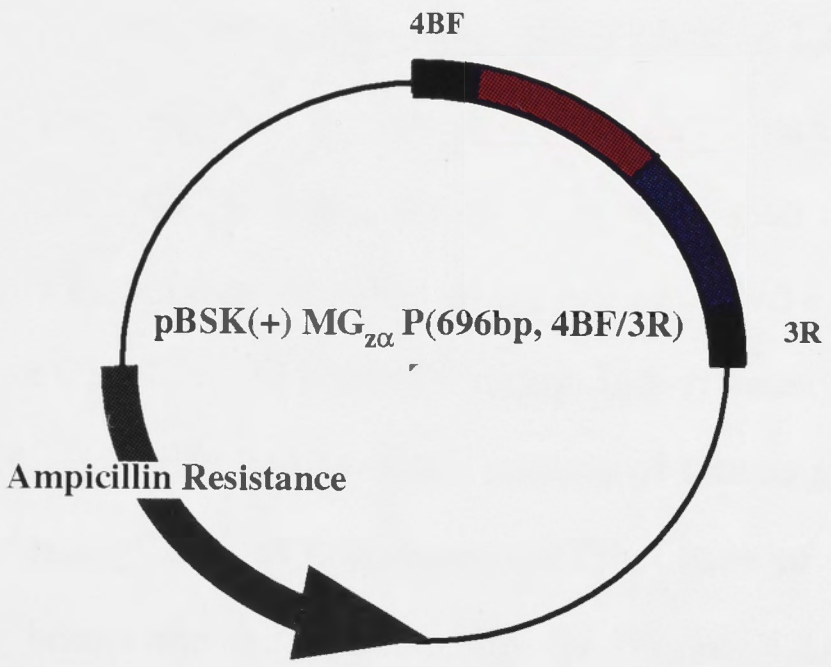
AMV Reverse Transcriptase
42°C for 1hr



Amplification through PCR

65 ° C annealing temperature
72 ° C extension temperature
30 sec extension time
TAQ Polymerase
35 cycles

Cloning and Verification



Part 4.2 Further Characterisation of $MG_{z\alpha}$.

Section 4.2.1 Introduction.

The previously cloned and sequenced partial $MG_{z\alpha}$ cDNA clone (Part 3.2, p65; Figure 3.5) was obtained using $RG_{z\alpha}$ and $HG_{z\alpha}$ consensus primers (4BF & 3R; Leck, 1993). These primers were designed to recognise highly conserved translated regions of the gene based on information gained from both the rat and human orthologous sequences. To amplify the complete open reading frame it would be necessary to design a primer to bind both the 5' and 3' untranslated region (UTR). Unfortunately, the same degree of conservation does not exist between the $RG_{z\alpha}$ $HG_{z\alpha}$ and $MG_{z\alpha}$ genes in either the 5' and/or 3' UTR's. It was, therefore, decided to isolate and characterise mouse genomic clones encompassing exon3 (~300bp) as well as any 3' UTR downstream of the 3R binding site. Once gained this sequence information would be combined with the known sequence of the 5' UTR (Part 3.2, p65) to design the primers needed to generate a the full length cDNA using an RT-PCR strategy (Sellner *et al.*, 1992; Section 2.3.10, p42).

Section 4.2.2 Attempts to Isolate $MG_{z\alpha}$ exon3.

a) Screening the Remaining $\lambda MG_{z\alpha}$ Genomic Clones with 3R. The $MG_{z\alpha}$P probe initially used to screen the C57BL/6 mouse genomic library spans both exon2 and exon3. Thus it was assumed that any λM clone isolated using this fragment would contain either one of the two translated exons. Thus the $\lambda MG_{z\alpha}$ clones obtained earlier (Part 3.2, p65) were revived through plate lysis (Section 2.2.2, p34). Each phage sample was then diluted and the DNA transferred to PCNM in concentrated 'dots' (Section 2.4.3, p45). The membrane-bound DNA was then subjected to high stringency hybridisation (63°C; Section 2.4.3, p45) with endlabelled 3R (Section 2.4.1, p44). It was hoped that this approach would allow us to rapidly identify those clones which encompassed exon3 without the need to rescreen the entire C57BL/6 λM genomic library. Unfortunately, 3R did not hybridise to any of the $\lambda MG_{z\alpha}$ genomic clones or the sample of mouse genomic DNA run as an independent 'control'. This was despite the fact that each of the other 3R containing controls clearly bound the radiolabelled primer (Figure 4.2A). This result was not,

however entirely unexpected because of the potential difference between rat, human and mouse copies of the 3R primer binding site (compare with 4BF; Section 3.2.5, p70). A subtle difference may affect the stability of the primer during hybridisation without affecting the RT-PCR strategy initially used to generate the $MG_{z\alpha}P$ probe. This is because the primer itself only needs to bind specifically once to generate the first strand. However, when the hybridisation was repeated under low stringency conditions (55°C) all of the $\lambda MG_{z\alpha}$ clones bound the labelled primer, including those which were previously found not to bind 3R (Section 3.2.3, p65). Later it would be shown that none of the $\lambda MG_{z\alpha}$ clones possessed copies of exon3. At this stage, however, several questions remained unanswered. Was the phage titre sufficient to produce a strong enough signal and if interspecies differences did exist between the 3R primer and the putative 3R mouse primer binding site how did they affect its binding? To answer these questions it was necessary to sequence a subclone of the mouse genome containing exon3 using a method which was independent of 3R. Therefore, the decision was made to clone the exon3 specific portion of the $MG_{z\alpha}P$(mimic) which could be used to verify the presence of exon3 (Figure 4.3).

b) Cloning $MG_{z\alpha}$ exon3 and exon2 Specific Fragments. The $MG_{z\alpha}P$(mimic) clone (Figure 4.3) was prepared, digested (2μg) with *Sac*I and the exon3 specific fragment separated from the rest of the vector using agarose (1%) gel electrophoresis. The DNA band was then gel purified, digested with *Hin*f1 which cuts downstream of the putative exon2/3 boundary and blunt ended. The resulting fragments were then ligated into *Sma*I cut pBSK(+), confirmed through restriction analysis and sequenced using the universal forward and/or reverse primers (Figure 4.3; Part 2.6, p52). Note that the remainder of the vector possessing part of exon2 was also recovered, religated and sequenced.

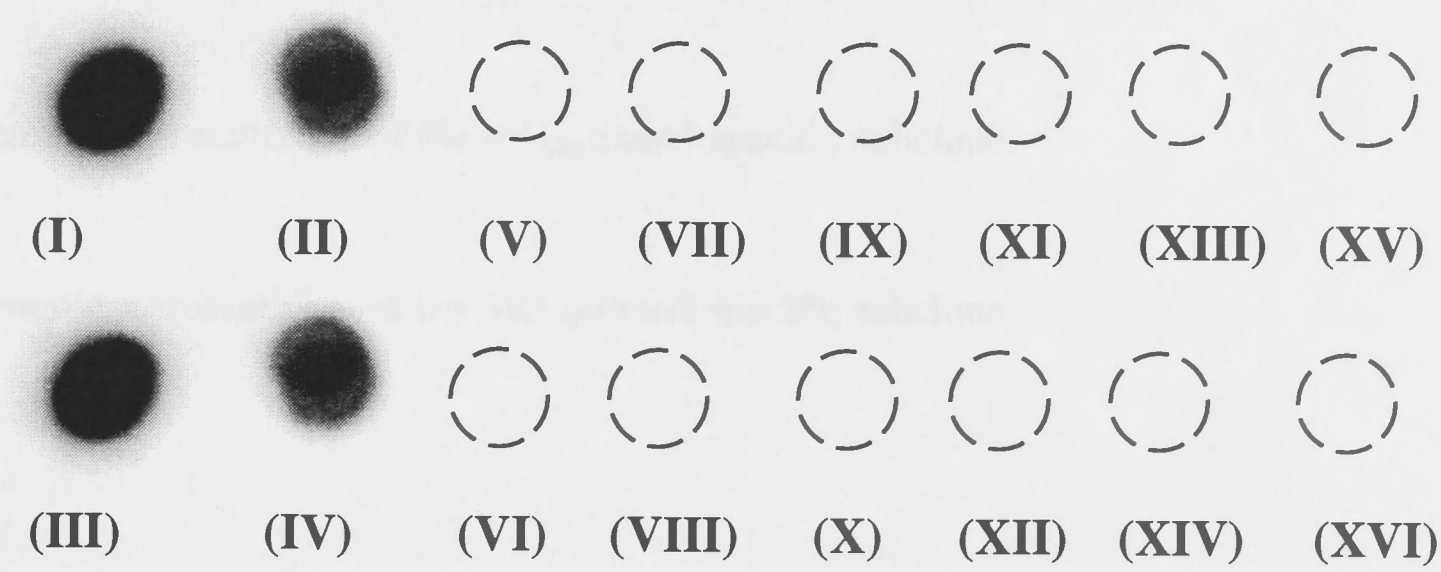
c) Screening $\lambda MG_{z\alpha}$ Genomic Clones with Exon Specific Probes. Both exon2 and exon3 specific fragments were gel purified, radiolabelled (Section 2.4.1, p44) and hybridised (65°C; Section 2.4.3, p45) with the, $\lambda MG_{z\alpha}$ dot blot. However, whilst each of the $\lambda MG_{z\alpha}$ clones bound the exon2 specific probe (Figure 4.2B), none of the 20 or so genomic clones isolated with the exon2/3 $MG_{z\alpha}P$ clone (Leck, 1993) bound the cloned portion of

Figure 4.2 Autoradiograph Analysis of $\lambda MG_{z\alpha}$ Genomic Clones.

(A) Autoradiograph of $\lambda MG_{z\alpha}$ genomic blot, following hybridisation with 3R. The positive controls for 3R, include: (I) a copy of the full length human cDNA; (II) pBSK(+)$HG_{z\alpha}$cDNA(F) (Matsuoka *et al.*, 1990); (III) the $MG_{z\alpha}$P clone (Leck, 1993); pBSK(+)$MG_{z\alpha}$P(696bp, 4BF/3R) and (IV) the $MG_{z\alpha}$P(mimic), a construct made by removing a 100bp fragment from the pBSK(+)$MG_{z\alpha}$P(696bp, 4BF/3R), and used as a control for RT-PCR (see also Figure 4.4; Leck, 1993). Positions (V) to (XVI) represent the $\lambda MG_{z\alpha}$ genomic clones isolated previously ($\lambda MG_{z\alpha}$1 to $\lambda MG_{z\alpha}$11 respectively; see Part 3.2, p65).

(B) Autoradiograph of $\lambda MG_{z\alpha}$ dot blot, following hybridisation with the exon2 specific cloned portion of $MG_{z\alpha}$P(mimic). Genomic clones shown include: (1) $\lambda MG_{z\alpha}$1; (2) $\lambda MG_{z\alpha}$1; (3) $\lambda MG_{z\alpha}$3; (4) $\lambda MG_{z\alpha}$4; (5) $\lambda MG_{z\alpha}$5; (6) $\lambda MG_{z\alpha}$6; (7) $\lambda MG_{z\alpha}$7; (8) $\lambda MG_{z\alpha}$8; (9) $\lambda MG_{z\alpha}$9; and (10) $\lambda MG_{z\alpha}$10.

A



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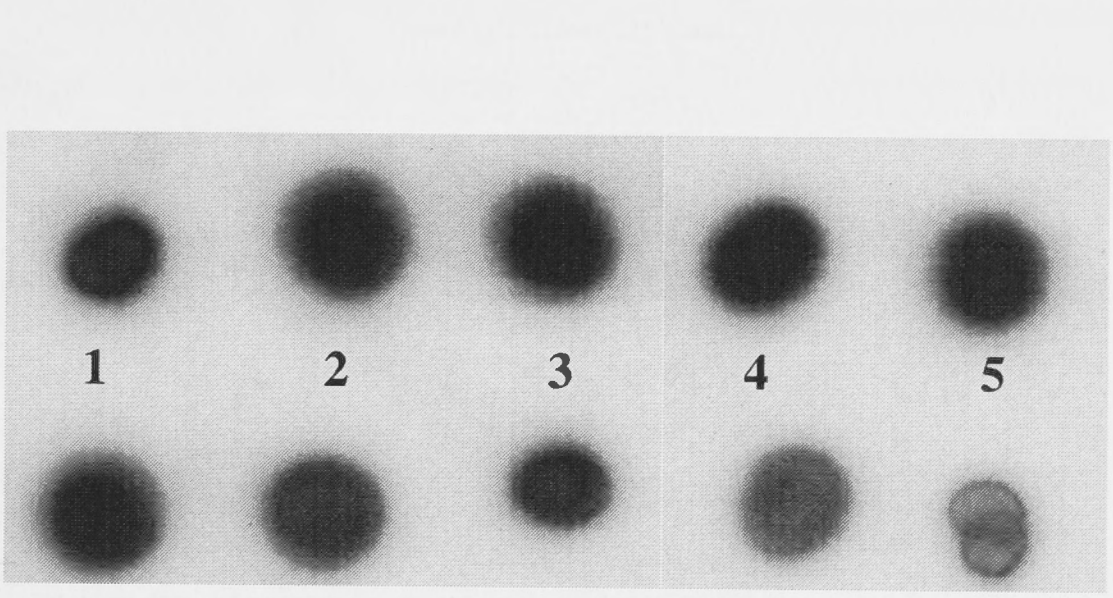


Figure 4.3 The $MG_{z\alpha}P$ Clone, Mimic & Exon Specific Subclones.

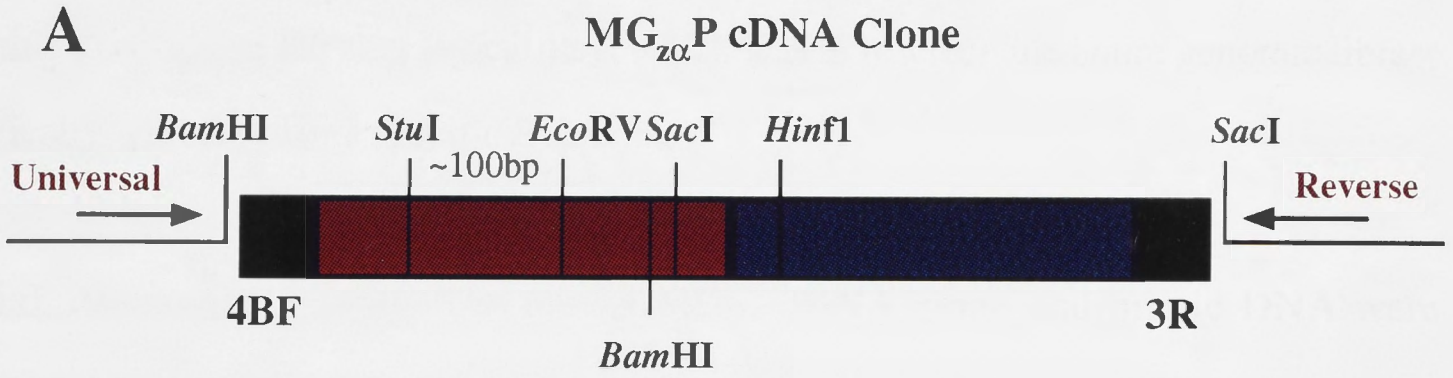
(A) Schematic representation of the full length partial $MG_{z\alpha}P$ cDNA clone (Leck, 1993).

(B) Schematic representation of the $MG_{z\alpha}P$(mimic), used to produce mRNA as a control in RT-PCR (Leck, 1993; Figure 4.4). Also shown are cloning and primer binding sites.

(C) Schematic representation of the $MG_{z\alpha}$ exon3 specific subclone.

(D) Schematic representation of the $MG_{z\alpha}$ exon2 specific subclone.

A


MG$_{z\alpha}$P cDNA Clone
*Bam*HI
*Stu*I
*Eco*RV *Sac*I
*Hin*f1
*Sac*I
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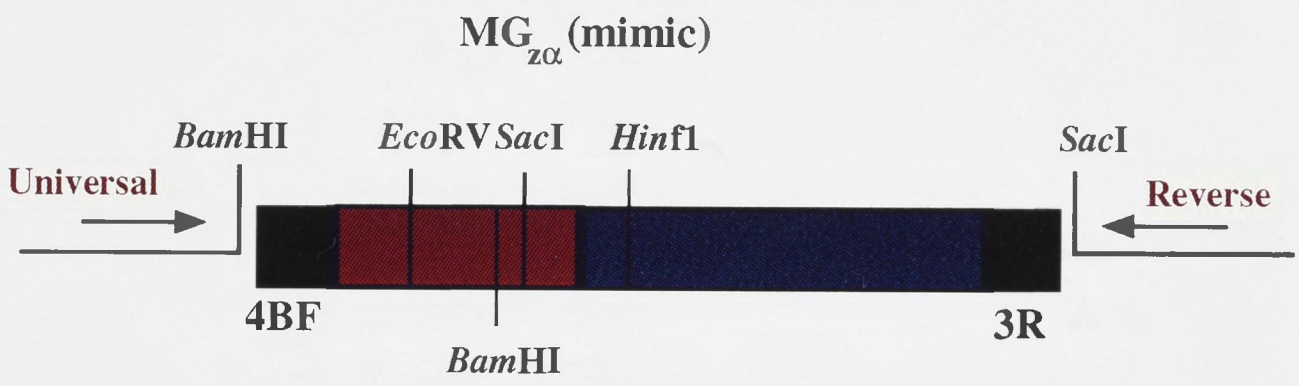
Universal
Reverse
4BF
3R
*Bam*HI

B


MG$_{z\alpha}$ (mimic)
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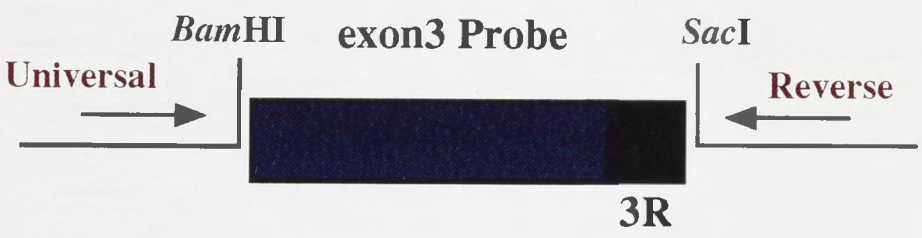
*Eco*RV *Sac*I
*Hin*f1
*Sac*I
Universal
Reverse
4BF
3R
*Bam*HI

C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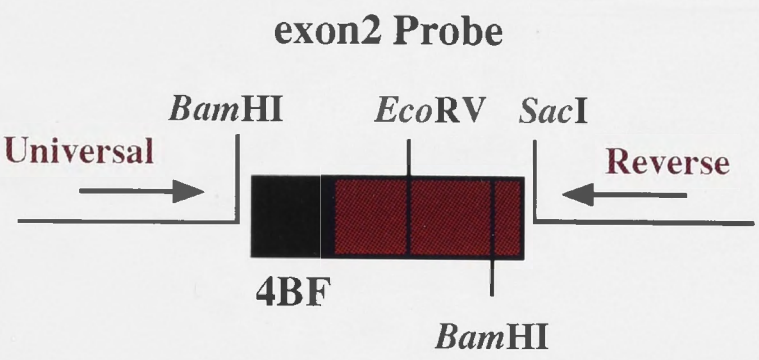
D


exon2 Probe
*Bam*HI
*Eco*RV
*Sac*I
Universal
Reverse
4BF
*Bam*HI

exon3, suggesting that they contain only $MG_{z\alpha}$ exon2. This may mean that the exon3 containing clones are under-represented (or that the exon2 containing clones are over-represented) in the mouse C57BL/6 genomic library. There was, however, insufficient time available to complete the next logical step, which was to rescreen the entire genomic library specifically with the exon3 specific fragment.

Special Acknowledgments. The partial $MG_{z\alpha}$ cDNA clone and mimic DNA were constructed by Kwong Joo Leck (Leck, 1993).

Part 4.3 Other Work.

Section 4.3.1 The use of RT-PCR to Obtain a $MG_{z\alpha}$ cDNA Clone.

a) Introduction. As none of the original $\lambda MG_{z\alpha}$ genomic clones possessed copies of exon3 (Part 4.2, p101) a different strategy was employed in an attempt to obtained a full length $MG_{z\alpha}$ cDNA. The two examples given below use oligonucleotide dT or modified oligonucleotide dT primers to synthesis the first strand and amplify putative $MG_{z\alpha}$ cDNA's.

b) From Total Mouse Cerebellum RNA. Mouse cerebellum RNA was prepared (Section 2.2.3, p35) and screened for the presence of $MG_{z\alpha}$ mRNA using the RT-PCR protocol developed to synthesise the partial cDNA clone (Leck, 1993; Figure 4.4A). In this instance an oligonucleotide dT (18mer) primer was used to prime the reverse transcriptase (RT) reaction. The 5' primer mG_zG_F was then used in conjunction with the oligonucleotide dT primer to amplify putative $MG_{z\alpha}$ cDNA fragments. However, due to the low annealing temperature of the oligonucleotide dT primer this procedure resulted in multiple bands (smears), which although they were cloned and sequenced did not contain $MG_{z\alpha}$ sequence.

c) From Mouse Cerebellum mRNA. The system next applied was adapted from the Promega RiboClone® System and uses a modified oligonucleotide dT primer [NOTI(Primer Adapter)PA; Table 2.1; Promega Catalogue] to prime the synthesis of the first strand, and a related primer (NOTPCR; Table 2.1) for subsequent PCR amplification. In this way the annealing temperature (T_A) during the PCR step can be raised, increasing the specificity of the 5' primer binding. The presence of the *Not*I recognition sequence at the end of the primer also assists in cloning PCR products. For the first strand synthesis, purified (Section 2.2.3, p35) mouse cerebellum mRNA (1.25µg) was added to a reaction mix containing AMV RT and the NOTIPA primer (Section 2.3.10, p42). The products of the first strand synthesis were then subjected to amplification by PCR using the mG_zG_F and/or mG_zG_F (*Not*I) 5' primers against the 3' NOTPCR primer. In this way a DNA band, corresponding in size to a predicted full length $MG_{z\alpha}$ cDNA clone (~1.5kb), was

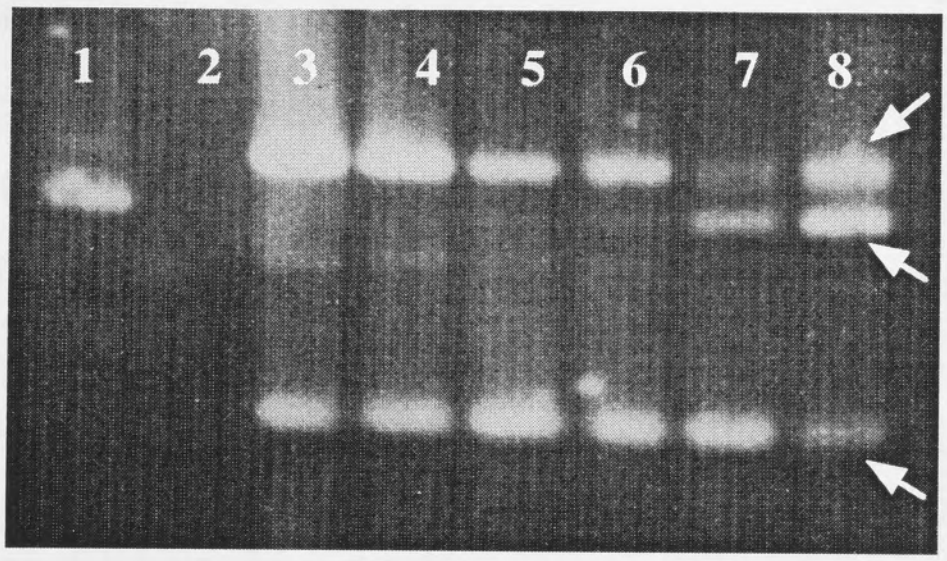
Figure 4.4 The Products of the Two RT-PCR Protocols.

(A) Each RNA preparation was diluted in DEPC treated ddH_2O and added to an RT-PCR mix containing both AMV RT and 'Super' TAQ DNA polymerase. The cycling conditions used were as follows: The first strand synthesis was conducted at 42°C for 59min. This was followed by a denaturing step (95°C, 5min) and the $MG_{z\alpha}$ specific cDNA (637bp) amplified using PCR: Cycles, 35x; ET, 30sec; T_E, 72°C; AT, 5sec; T_A, 63°C. This was followed by a final extension period of 5min at 72°C, after which the PCR product was heated (65°C, 15min) and separated by agarose (1%) gel electrophoresis: Lane 1, 10ng $MG_{z\alpha}P$(mimic); Lane 2, ddH_2O (control); Lane 3, 1 000ng RNA; Lane 4, 100ng RNA; Lane 5, 10ng RNA; Lane 6, 1ng RNA; Lane 7, 100pg RNA; Lane 8, 10pg RNA. Approximately 10pg of $MG_{z\alpha}P$(mimic) RNA (~500bp; Leck, 1993) is also present in each of the lanes as an RT control. In Lanes 7 and 8 it appears below the 696bp $MG_{z\alpha}P$ cDNA clone. The small band (*) present in each lane is a non-specific product which is routinely amplified under these conditions.

(B) The product of first strand synthesis (RT reaction; Section 2.3.10, p42), using the Riboclone System NOTIPA was diluted in (1/20) within ddH_2O and subjected to PCR amplification (Section 2.3.9, p42). Cycles, 35x; ET, 2min; T_E, 72°C; AT, 30sec & T_A, 59°C. The product of each of the reactions was then run on an agarose (1%) gel: Lane 1, straight mix (RT) using mG_zG_F(NotI) and NOTPCR primer [mG_zG_F(NotI)/NOTPCR]; Lane 2, 1/20 RT mG_zG_F(NotI)/NOTPCR; Lane 3, RT mG_zG_F/NOTPCR; Lane 4, 1/20 RT mG_zG_F/NOTPCR; Lane 5, RT mG_zG_F/3R; Lane 6, 1/20 RT mG_zG_F/3R; and Lane 7 ddH_2O (control). Note the putative $MG_{z\alpha}$ cDNA 'fragment' (~1.5kb; Lanes 1 to 4).

(C) Restriction Analysis of the Putative $MG_{z\alpha}$ cDNA Clone. Once gel purified the putative $MG_{z\alpha}$ cDNA clone (250ng) was cut with one of a number of restriction enymes and run on an agarose (1%) gel: Lane 1, uncut product; Lane 2, *Bam*HI; and Lane 3, *Sac*I. Note that both *Bam*HI and *Sac*I failed to digest the 1.5kb fragment (Lanes 2 & 3).

A

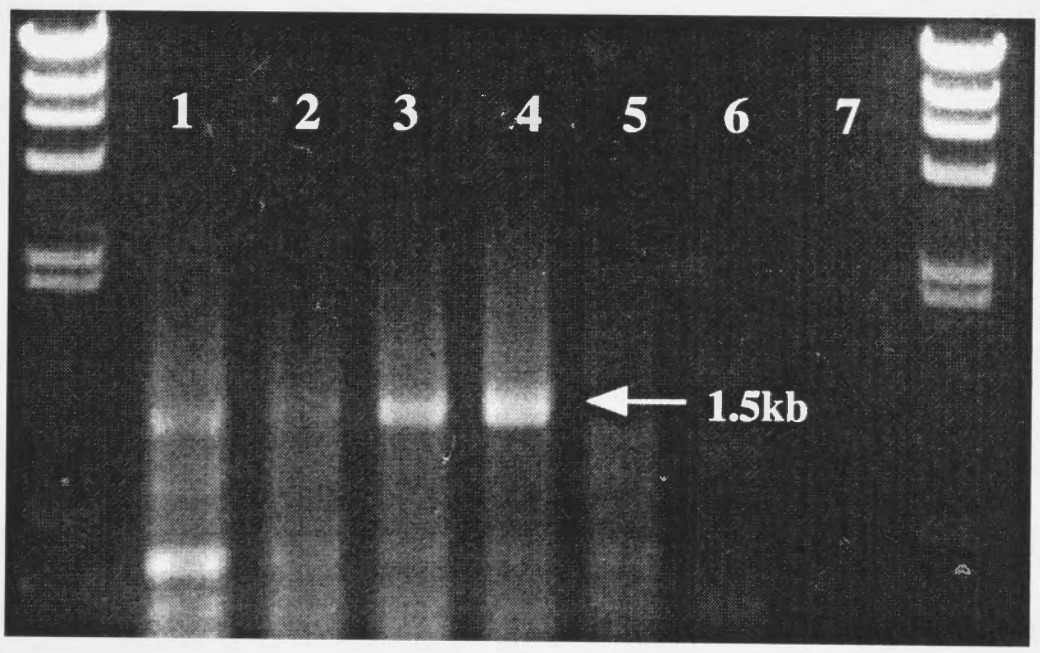


$MG_{z\alpha}P$(696bp, 4BF/3R)

$MG_{z\alpha}$(mimic)

(*)

B



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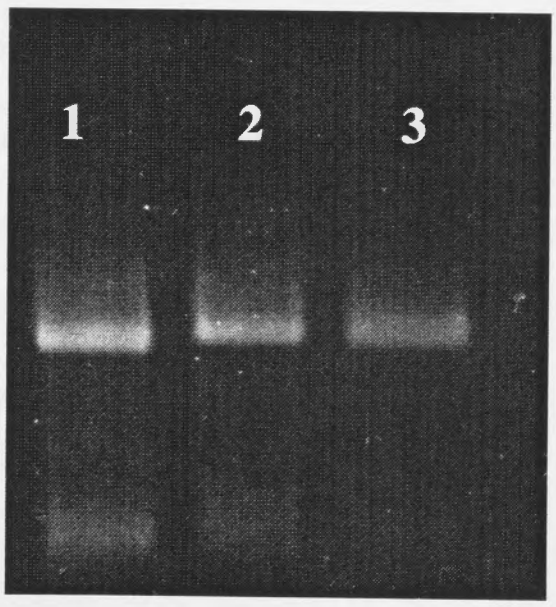
C



1.5kb

amplified. Because this result was reproduced at the higher more stringent annealing temperature (61°C) the band was gel purified and subjected to restriction analysis. Both *Sac*I and *Bam*HI recognition sequences present within the cDNA clone, however, this purified fragment proved to be resistant to digestion by either enzyme (Figure 4.4C). Furthermore, no band was amplified from the initial RT mix using the mG_zG_F/3R primer set, even though 3R has been consistently used to amplify the $MG_{z\alpha}P$ cDNA fragment from total RNA using the on-step RT-PCR reaction (Section 2.3.11, p43; Leck, 1993).

Section 4.3.2 Northern Analysis.

Many of the questions regarding a possible phenotype for a $MG_{z\alpha}$ knockout surround its tissue distribution which in turn may have bearing on how widespread and important its biological function might be. As mentioned previously there are several studies, including those using RT-PCR analysis, which suggest that $G_{z\alpha}$ may be present in a number of non-neuronal organs (e.g. the lungs, kidneys etc), however the specificity of these approaches is open to question because of the high degree of sequence similarity between the different members of this family (Section 1.2.2, p3). This proposition is supported by the amplification of an unknown product using specific $MG_{z\alpha}$ primers from mRNA isolated from a tissue known to be especially abundant in $MG_{z\alpha}$ (Section 4.3.1, p106). In an attempt to answer these questions both the exon2 and exon3 specific fragments were used in northern analysis. Whole cell RNA was prepared from both cerebral hemispheres, mid brain, cerebellum, kidneys, lungs, liver, sciatic, heart, hind brain, spleen and the yield determined. 10µg of each sample was then run on an agarose (1%) gel (Section 2.4.4, p47). The RNA was then blotted onto PCNM (Northern Transfer) and hybridised (55°C) with both $MG_{z\alpha}$ exon2 and $MG_{z\alpha}$ exon3 specific probes (Part 4.2, p101). Unfortunately, both radiolabelled fragments failed to bind a band within the expected size range in either the neuronal and/or non-neuronal tissue samples. This result is difficult to accept because of the abundance of $MG_{z\alpha}$ message within neuronal tissues (Hinton *et al.*, 1990), however, time constraints meant that this experiment could not be repeated.

CHAPTER 5 General Discussion.

Part 5.1 Introduction.

The work presented in this thesis outlines the approach taken to target $G_{z\alpha}$ in ES cells *in vitro*, however, the implications of disrupting this gene in the whole animal remain unresolved. Previous studies have shown that gene dosage effects, cross talk and functional redundancy have the potential to contribute to the overall phenotype of a knockout and in so doing may mask the true biological significance of the targeted gene product (Erickson, 1993; Shastry, 1994). Such studies have also demonstrated that the data gathered from gene knockouts is interpreted best if accompanied by corroborating and independent evidence. In this chapter I will discuss the rationale for gene targeting as an approach for further understanding the biological function(s) of $G_{z\alpha}$, including the potential problems and possible outcomes.

Part 5.2 Targeting Gene Expression: Past Outcomes.

Section 5.2.1 Introduction.

Previous studies have revealed that mice which possess a disrupted gene product can express a number of different phenotypes. In many instances knockout mouse are either phenotypically normal (Hooper *et al.*, 1987; Kuehn *et al.*, 1987; Bueler *et al.*, 1992; Clarke *et al.*, 1992; Collins & Wilson, 1992; Snouwaert *et al.*, 1992), produce a minimal phenotype (Zijlstra *et al.*, 1990; Joyner *et al.*, 1991; Donehower *et al.*, 1992), or express a phenotype in unexpected tissues (Shastry, 1994). When the observed response to the absence of a particular gene product is specific, obvious, non-lethal and if it correlates with known activity and tissue distribution then a biological function can easily be assigned to the protein. However, if the disrupted gene is required for survival of the foetus or if no obvious phenotype is expressed following the removal of the protein then very little can be deduced about its biological function (see below).

Section 5.2.2 Lethal Outcomes.

In laboratory reared mice the naturally occurring rate of prenatal death is about 20-25% (Wilmut *et al.*, 1986). This can be greatly increased through the introduction of lethal genetic mutations and is dependent upon whether or not the protein that has been 'knocked out' plays an indispensable role in the survival of the foetus during development (Gridley *et al.*, 1987; Green, 1989; Copp, 1995). Although gene lethal outcomes make it difficult to assign a biological function to the targeted protein, its role at a particular stage of foetal development can still be determined (Copp, 1995). Furthermore, to avoid the potentially lethal outcome of suppressing the ubiquitously expressed $G_{i2\alpha}$ *in utero*, the *in vivo*, tissue specific, post natal expression of antisense $G_{i\alpha}$ RNA has been used to disrupt the translation of $G_{i2\alpha}$ mRNA (Simonds *et al.*, 1989; Lyons *et al.*, 1990; McKenzie & Milligan, 1990; Moxham *et al.*, 1993). In contrast, the localisation of $G_{z\alpha}$ seems to suggest that it has a restricted function which is not linked to the survival of the foetus. This is further supported by the observation that the CNS appears to have little or no survival value *in utero* (Copp, 1995).

Section 5.2.3 Functional Redundancy.

If a gene product has even a small functional advantage then it will be retained during evolution. It is not surprising, therefore, that many knockout mice do not differ greatly from the wild-type. It is not, however, correct to assume that a minimal or non-existent phenotypic change is due to the removal of a biologically insignificant protein. This is because in some instances there may be a facility *in vivo* to compensate for the loss of a functionally important protein by substituting another in its place (Brookfield, 1992; Tautz, 1992). The best example of this sort of functional redundancy is seen in the retanoic receptor α1 (RARα1) knockout mouse. RARα1 is the most abundant and conserved of the retanoic receptor isoforms, which could be interpreted as evidence for an important biological function. Furthermore, the specific ligand for RARα1, retanoic acid has been strongly implicated in embryonic development (Lammer *et al.*, 1985; Moris Kay, 1993). Despite such observations, however, RARα1 knockout mice develop normally (Li En *et al.*, 1993). It has been proposed that the lack of a severe phenotypic change in these mice may be due to the recruitment of other retanoid receptors which could act to compensate for the loss of functional RARα1 (Li En *et al.*, 1993). It is also possible that RARα1 may not be required for development at all, but may fulfil some other biological role. Further examples of minimal phenotypic changes arising as a consequence of the disruption of apparently 'indispensable' genes include, p53 (Gammon & Lane, 1987; Marshall, 1991; Donehower *et al.*, 1992), PKCγ (Abeliovich *et al.*, 1993), the homeobox (Hox) genes, hox-3.1 (LeMouellic *et al.*, 1992), hox-1.5, hox-1.6 (Chisaka & Capecchi, 1991; Lufkin *et al.*, 1991; Chisaka *et al.*, 1992) and c-abl (Tybulewicz *et al.*, 1991; Schwartzberg *et al.*, 1991). In these instances several related genes may need to be disrupted in the same animal to circumvent what may be, functional redundancy in these knockouts. Whilst it is not yet possible to predict whether functional redundancy will play a role in the phenotype expressed by a $G_{z\alpha}$ knockout mouse, the relatively restricted tissue distribution of $G_{z\alpha}$ suggests that its biological role is likely to be both unique and one which is not easily fulfilled by a functionally similar gene product (e.g. another G-protein; note that tissue distribution is not always the best guide to the *in vivo* function of a protein; Section 5.2.4, p113).

Section 5.2.4 Unexpected Phenotypes.

Whilst it may appear that knocking out $G_{z\alpha}$, which is abundant within the nervous system, may lead to developmental defects within these tissues, there are many examples in which knocking out a gene produces unexpected phenotypic changes. These include disruptions to the genes encoding myf-5 (Braun *et al.*, 1992; Rudnicki *et al.*, 1992), transforming growth factor α (TGFα, Luetteke *et al.*, 1993; Mann *et al.* 1993), transforming growth factor β1 (TGFβ1, Shull *et al.*, 1992), c-Src (Soriano *et al.*, 1991) and NGF (Lee *et al.*, 1992). In each instance it was expected that the absence of these proteins would result in serious developmental abnormalities, corresponding with the critical role that each gene product is thought to play in several restricted tissue types: Myf-5, myogenesis and skeletal muscle differentiation; TGFα: angiogenesis, induction of cell migration and control of cell proliferation; TGFβ, cell proliferation, differentiation, extracellular matrix protein production, cell adhesion and angiogenesis (Bernard *et al.*, 1990); c-Src, found in platelets and the brain, is implicated in cell division and signal transduction; and NGF (Part 1.3, p13). However, when each of these genes are knocked out *in vivo*, the mutant differs only slightly from that of the respective wild-type, with phenotypic changes appearing in unexpected tissues of the body: Myf-5 knockout mice have abnormal rib development; TGFα knockout mice are susceptible to corneal inflammation and have 'curly' hair; TGFβ knockout mice have no obvious developmental defects except for multifocal inflammatory disease; and although c-Src deficient mice develop serious osteoporosis (suggesting that this protein may have some function in osteoclast integrity; Nowak, 1991; Horn *et al.*, 1992) they have normal brain and blood. It is difficult to accept that in all of these cases functional redundancy (Section 5.2.3, p112) is the sole reason for a lack of more serious developmental abnormalities. It has been proposed, and what these observations suggest, is that some proteins serve important functions in tissues, other than those in which they are abundantly expressed (Shastry, 1994; Appendix 3.1). This also means that assigning a biological role, to any protein, based solely on its localisation can be misleading. It will, therefore, be interesting to see if any of the functions assigned to $G_{z\alpha}$, based on its tissue distribution, prove to be correct following examination of the $G_{z\alpha}$ knockout (Part 1.4, p23).

Part 5.3 Cross Talk and Other Considerations.

For phenotypic changes to occur, in tissues that are seemingly unrelated to the targeted gene product, a form of cross talk may be occurring which affects the regulation of other genes in the body during development (Erickson, 1993; Shastry, 1994; Gerlai, 1996). This theory is supported by the observation that in man a single mutation can give rise to a large variety of abnormalities (Davies, 1993). Observed phenotypic changes may also be due to: (i) the effect that the insert has on the transcription of adjacent genes (e.g. polar effects); and/or (ii) the genetic background of the knockout animal (Shastry, 1994; Gerlai, 1996; Figure 5.1). Therefore, to account for each of these effects, and in so doing produce adequate control animals, it may be necessary to either: (i) rescue the mutation by introducing a transgene that expresses the functional protein (Chapter 4, p98) and/or (ii) generate a 'knockin' mouse (control) by introducing a small DNA marker, flanking the gene of interest, without disrupting its expression. If, however, the background of the targeted ES cells and the mouse strain to which the chimæra is mated are not identical (Figure 5.2; Gerlai, 1996), then using one, or other, of the parent strains as a control for this 'hybrid' knockout animal may not be adequate. This consideration would seem to be particularly important when targeting genes thought to be involved in the more subtle aspects of biological function, such as learning and memory (Gerlai, 1996; Appendix 5.1). Several examples include: PKCγ (Abeliovich *et al.*, 1993; Chen *et al.*, 1995); the NMDA receptor (ε1; Sakimura *et al.*, 1995); and the dopamine receptor (d2; Balk *et al.*, 1995). The $MG_{z\alpha}$ chimæra (Figure 3.21) are currently being bred with C57BL/6 mice, as the targeted ES cells were derived from a C57BL/6 mouse strain. However, if the knockout had been made using 129 ES cells, then it would be necessary to backcross the resulting hybrid animal several times to increase the proportion of one of the inherited population of genes (e.g. 9-10 backcrosses will reduce the proportion of one of the inherited set of sequences to approximately 1% of the mouse genome; Festing, 1992). Lastly, it is of course possible that a failure to observe an obvious phenotypic change, or one which supports previous information, may simply be due to the lack of a correct *in vitro /in vivo* experimental challenge that would reveal/expose the deficiencies caused by removal of the gene product (e.g. IL-5; Kopf *et al.*, 1996).

Figure 5.1 Behavioural Differences Between Mice Strains 129 & BL/6.

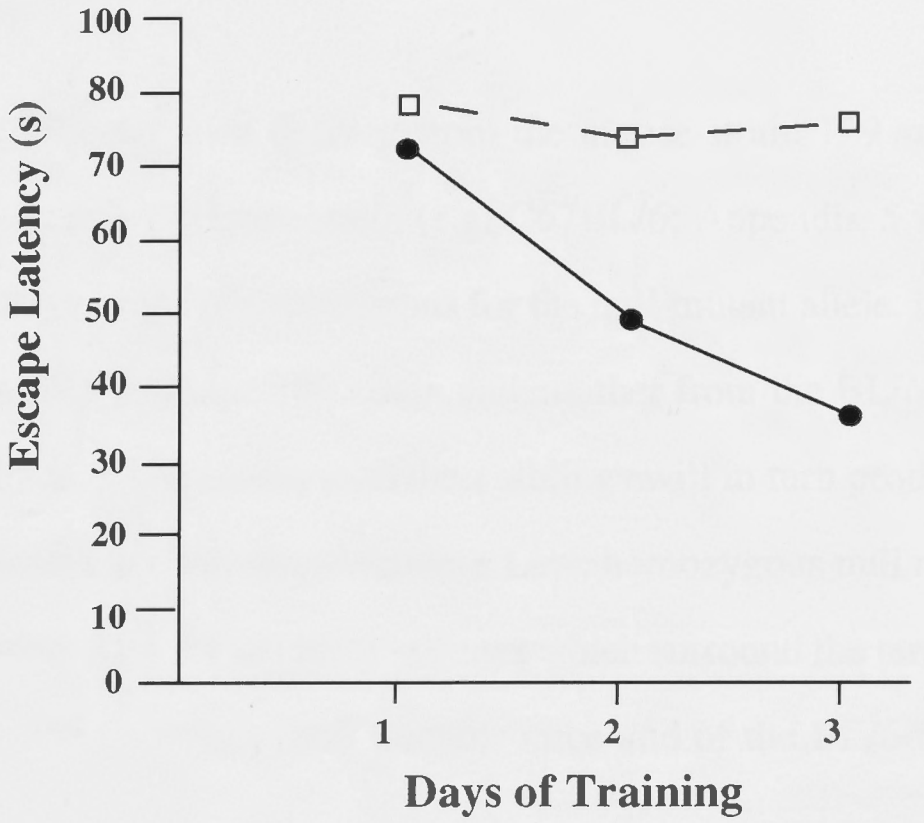
(Taken from Gerlai, 1996)

(A) Spatial Performance Learning in the Water Maze (Bernasconi-Guastalla *et al.*, 1994; Wolfer & Lipp, 1994; Gerlai, 1996). The escape latency, or the time required to locate and climb onto a hidden platform submerged 5mm below water, against the days of training. Although both 129 (open squares; n=41) and BL/6 (black circles; n=61) mice started with the same level of performance, BL/6 mice achieve a significantly better performance with training.

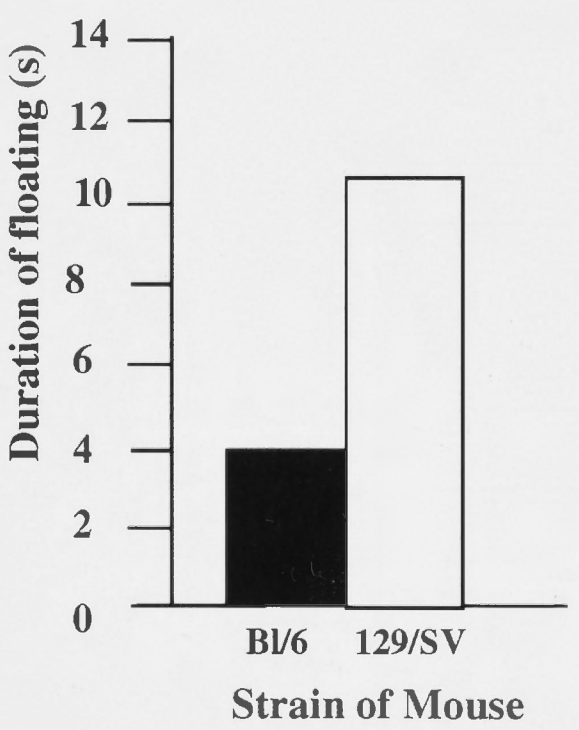
(B) Duration of floating in the water maze (see above). Shows a significant difference in the average duration of floating between 129 and BL/6 mice strains (Gerlai, 1996).

(C) The locomotion score in the water maze or the exploratory activity. 129 mice exhibit a significant hypoactivity (or decrease locomotion score) when compared to BL/6 mice (Gerlai, 1996).

A


Escape Latency (s)
100
80
70
60
50
40
30
20
10
0
1
2
3
Days of Training

B


Duration of floating (s)
14
12
10
8
6
4
2
0
Bl/6
129/SV
Strain of Mouse

C

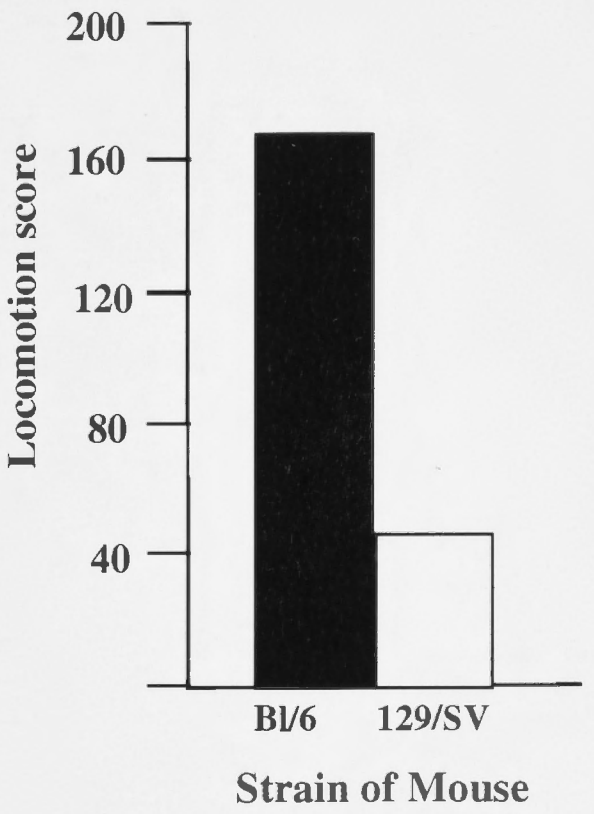
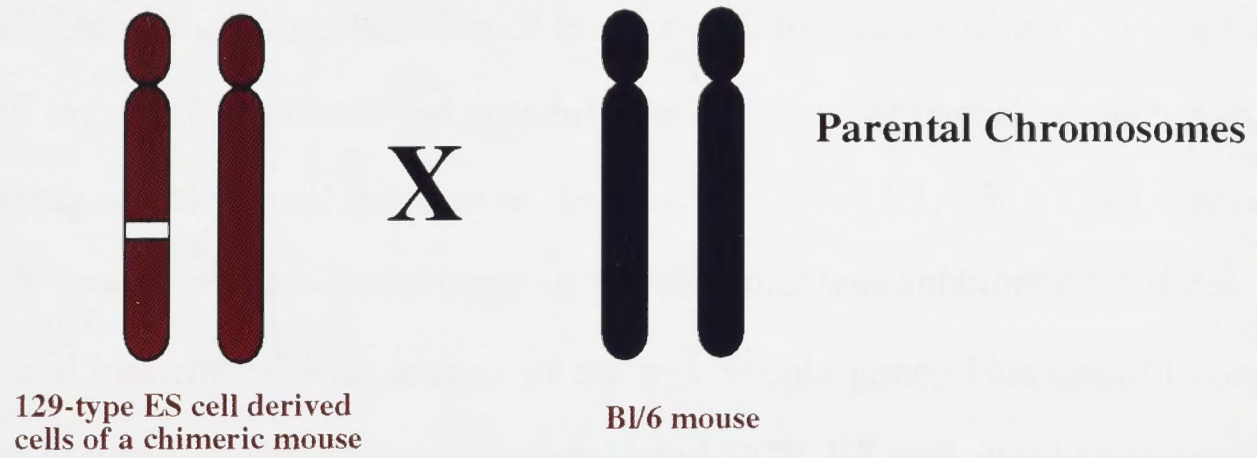
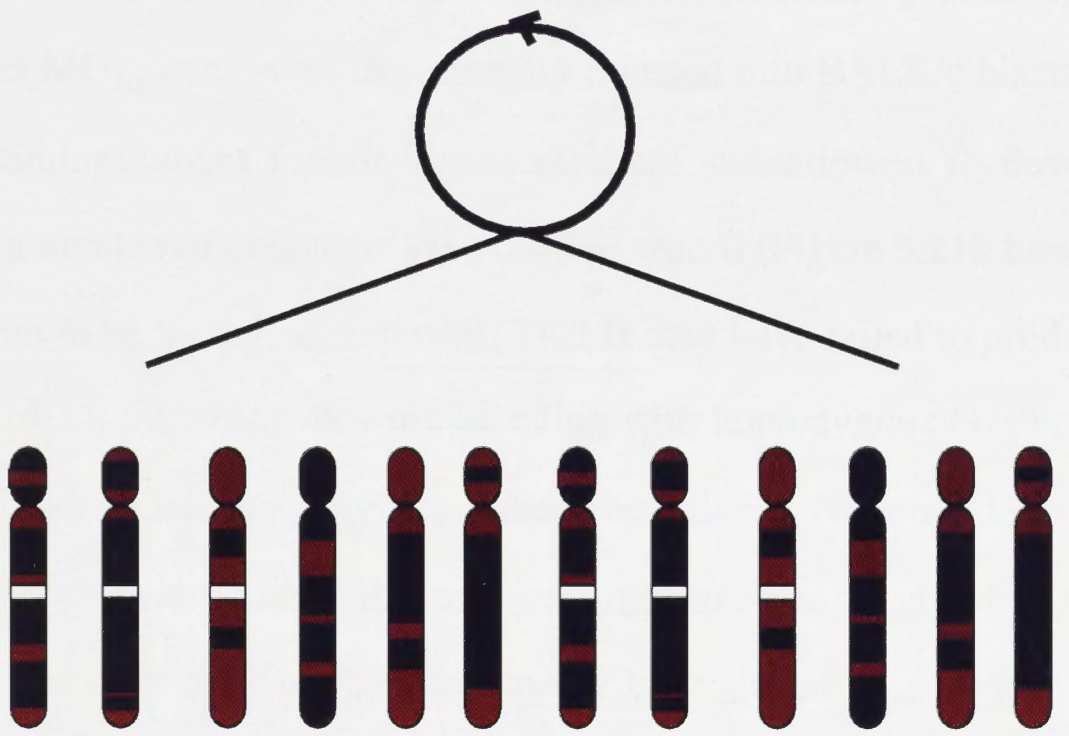

Locomotion score
200
160
120
80
40
Bl/6
129/SV
Strain of Mouse

Figure 5.2 Chromosomal Constitution of Mice Generated by Gene Targeting.

(Taken from Gerlai, 1996; see also Part 3.1, p56)

If the targeted ES cells were derived from the mouse strain 129 and the chimæra were mated to mice from a different strain (e.g. C57BL/6; Appendix 5.1). The offspring (F1 generation) will not only be heterozygous for the null-mutant allele, but will also have one set of chromosomes from the 129 strain and another from the BL/6 mouse strain. These heterozygous mice, when mated with their siblings will in turn produce a segregation F2 population in which according to Mendel's Law, homozygous null mutant and wild-type mice will be found. This means that the genes which surround the targeted locus will be of the 129-type in the knockout (null mutant) mice and of the BL/6-type in the wild type mouse (control).





Recombinant F2 Chromosomes

Part 5.4 Summary.

This report has discussed the steps taken to knock out the G-protein, $G_{z\alpha}$ *in vivo*. Initially it was decided to disrupt the expression of the gene as close as possible to the start site of transcription in order to eliminate the possibility of truncated or partial products surviving and performing a background function in the knockout (Part 3.1, p56). Thus, a region of the C57BL/6 mouse genome encompassing $MG_{z\alpha}$ exon2 was subcloned (Part 3.2, p65), sequenced and interrupted with a copy of the pgkNeopla gene. This control construct would later be used as the control for the nested PCR ES cell identification of true homologous recombinants, as it resembles the $G_{z\alpha}$ gene region following targeted inactivation with the pgkNeopla marker. To create the targeting construct a portion of the control construct encompassing endogenous primer binding sites was replaced with the pgkTKpla gene. This plasmid was then used to inactivate the $G_{z\alpha}$ gene in an ES cell line (C57BL/6) chosen to increase the identity and, therefore, the frequency of homologous recombination between the targeting construct and the host genome following electroporation (Part 3.3, p86). Heterozygous ES cell clones possessing a single deleted copy of the $MG_{z\alpha}$ gene were then directly injected into BALB/c blastocysts, implanted within pseudopregnant female foster mothers and allowed to develop. Using this technique a number of chimæras have been produced (Figure 3.21), however, as yet none have proven to be 'germline' chimæra. That is they have failed to produce heterozygous null (-/+) $MG_{z\alpha}$ mutants following breeding with homozygous (+/+) positive C57BL/6 partners, although it is too early to say that they will not. Not only do the foreign ES cells have to compete with those of the inner cell mass to enter the germline (see also Part 3.1, p56), but that only 50% of the germline cells produced (e.g. sperm) will possess the disrupted (or knocked out) copy/allele of the gene. Finally, steps were taken to isolate the third exon of $MG_{z\alpha}$ so that the sequence information gained by sequening this region of the gene could be used to create a full length $MG_{z\alpha}$ cDNA. An $MG_{z\alpha}$ exon3 specific probe was isolated and used to rescreen the $\lambda MG_{z\alpha}$ cDNA clones isolated using the exon2/3 partial cDNA fragment (Leck, 1993). Although none of these genomic clones encompass the exon (Part 4.2, p101), the cloned fragment containing part of exon3 can later be used to rescreen the entire genomic library.

Part 5.5 Conclusion.

The putative biological functions for $G_{z\alpha}$, including a proposed role in cytosolic signalling, remain unclear primarily because only circumstantial evidence is currently available (e.g. tissue distribution, *in vitro* associations etc; Part 1.4, p23). The development of a $G_{z\alpha}$ knockout mouse would allow such hypotheses to be directly tested and assist in elucidating the significance of this enigmatic G-protein.

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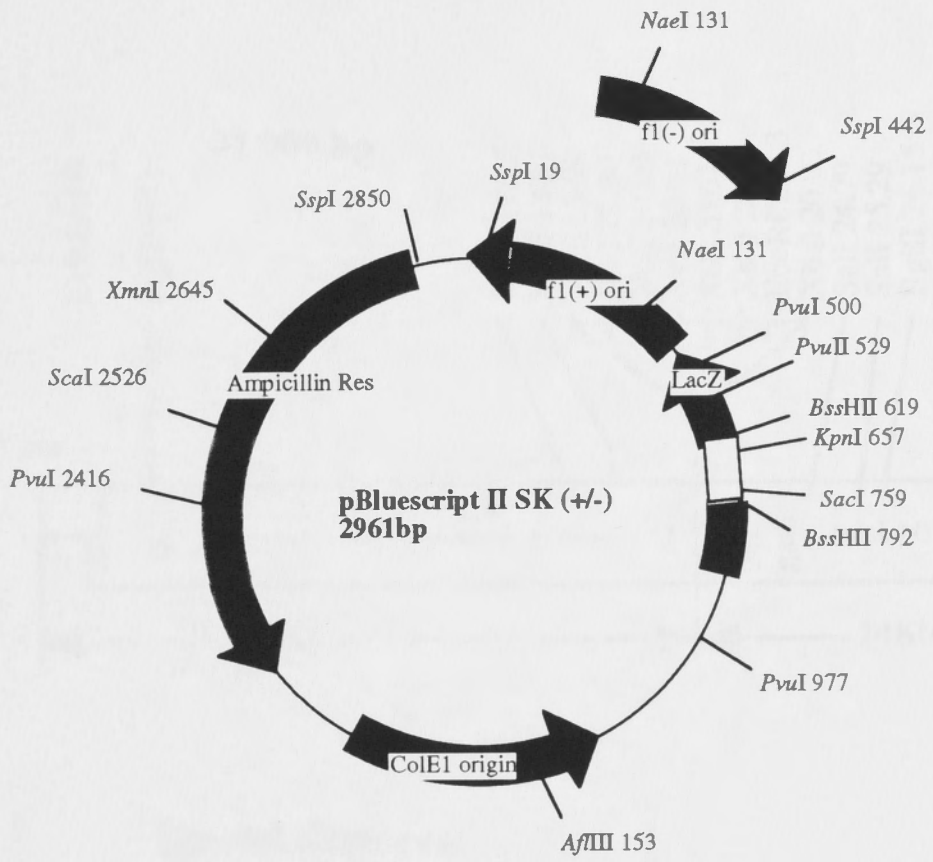
APPENDICES

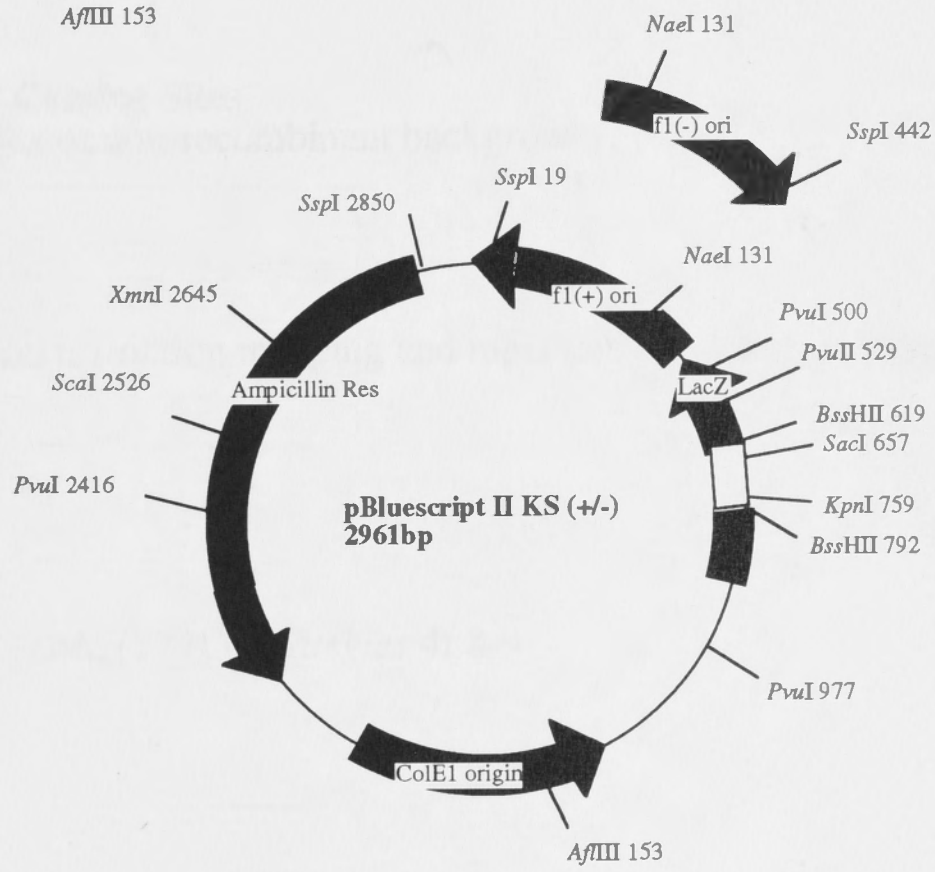
Appendix 1.1 Standard IUPAC One-Letter Amino Acid Codes.

A	Alanine
C	Cysteine
D	Aspartic Acid
E	Glutamic Acid
F	Phenylalanine
G	Glycine
H	Histidine
I	Isoleucine
K	Lysine
L	Leucine
M	Methionine
N	Asparagine
P	Proline
Q	Glutamine
R	Arginine
S	Serine
T	Threonine
V	Valine
W	Tryptophan
Y	Tyrosine.

Appendix 2.1

pBluescript Cloning Vectors

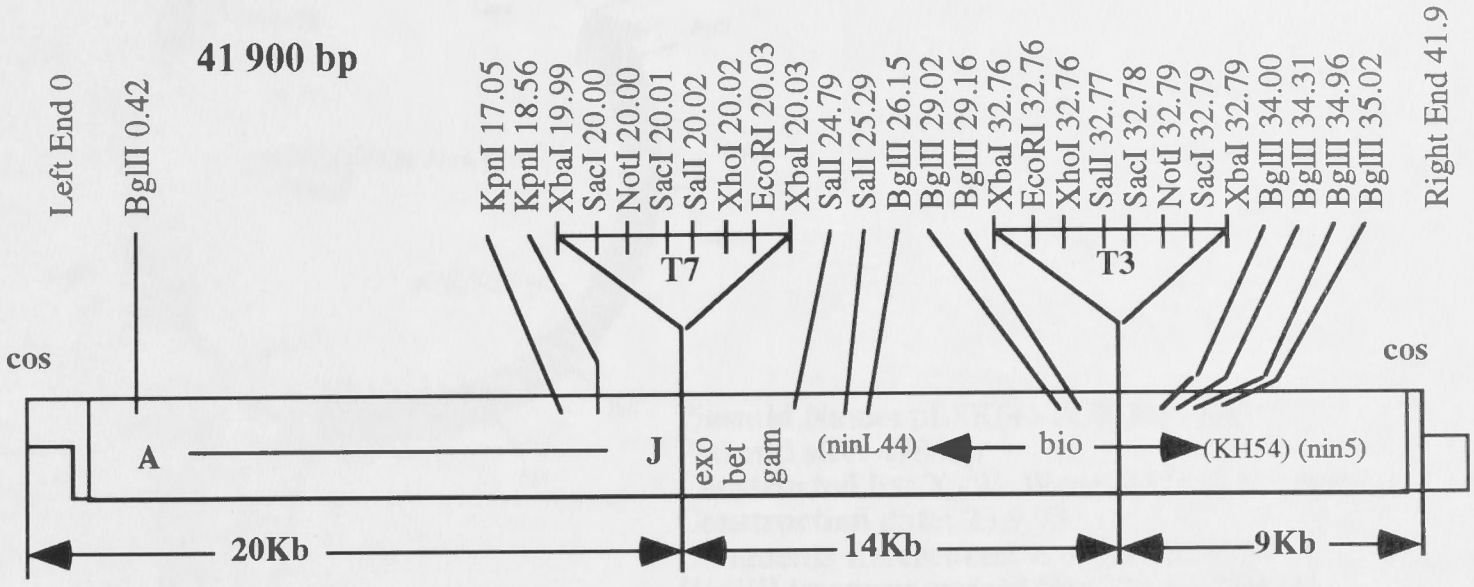




Plasmid Names: A. pBluescript II SK(+/-) & B. pBluescript II KS (+/-)
Plasmid Size: 2961bp
Comments: Taken from p326 Stratagene Technical Manual
Kindly provided by: Dr K. Matthaei

Appedix 2.2

Lambda DASH II Vector



Special Features:

Increased number of Cloning Sites
Spi+/P2 selection reduces non-recombinant background

Applications:

Allows high resolution restriction mapping and rapid chromosomal walking

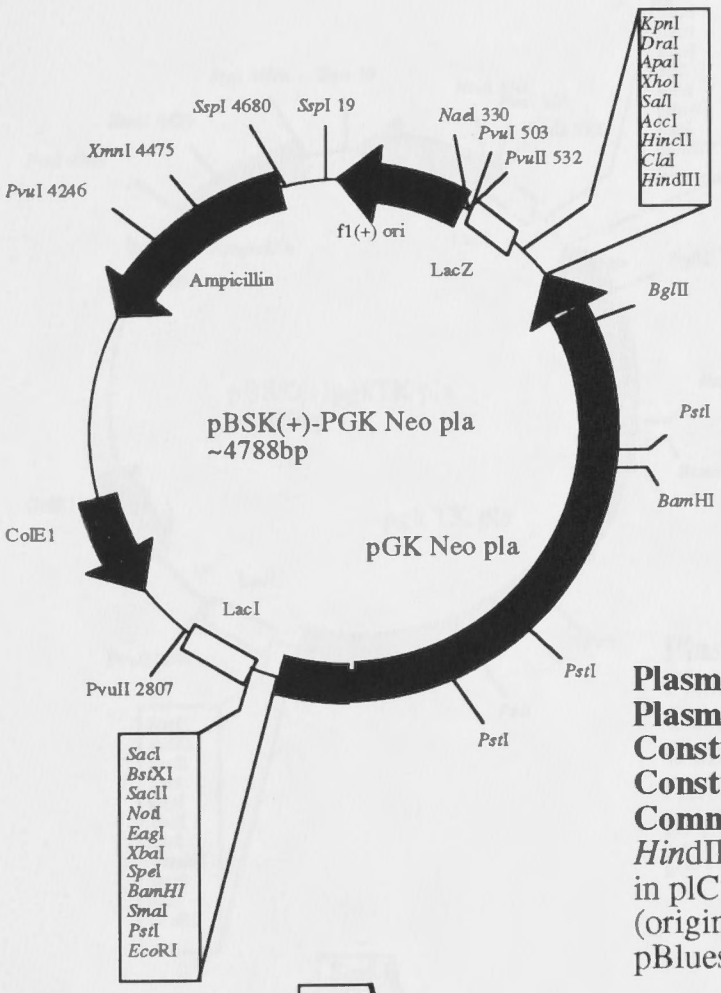
Cloning Capacity:

Capacity of 9-23Kb

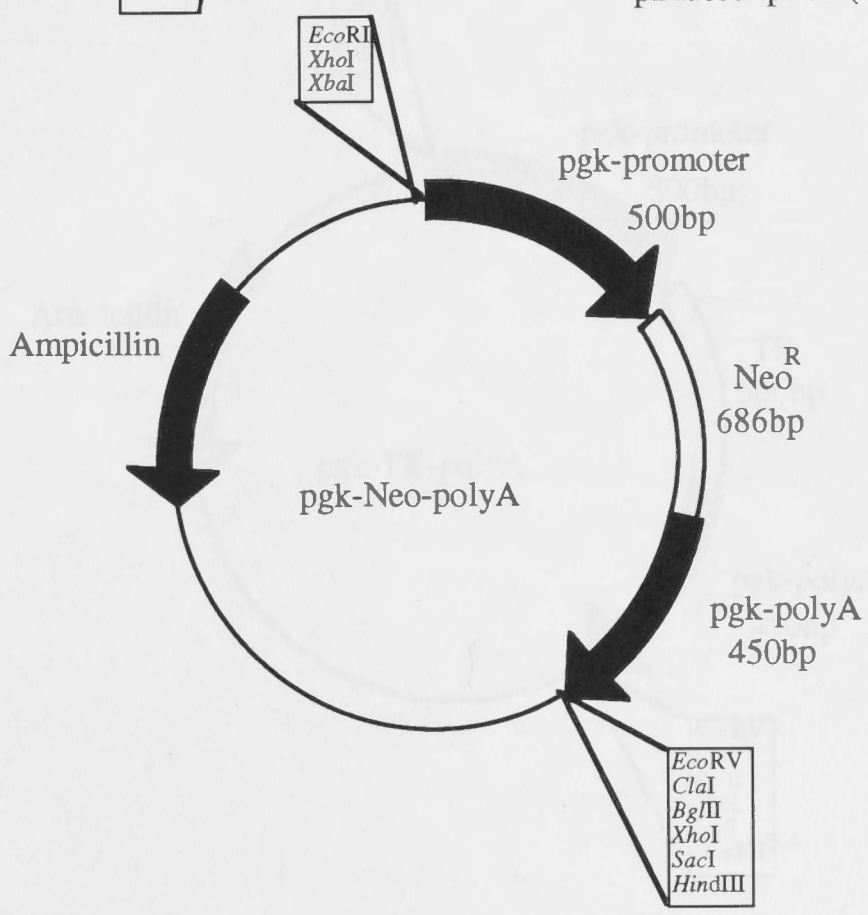
Reference Elgin, E., *et al.*, (1991) *Strategies* **4:** 8-9

Appendix 2.3

pgkNeo Cloning Vectors



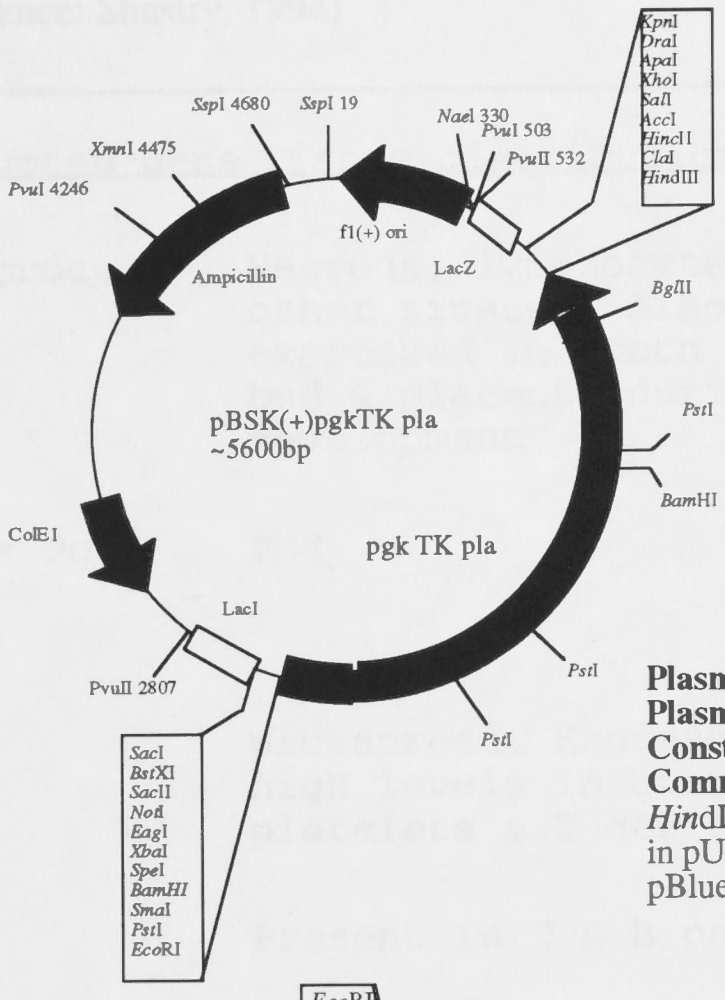
Plasmid Name: pBSK(+)-PGK Neo pla
Plasmid size: 4788bp
Constructed by: X.-W. Wang & Dr K. Matthaei
Construction date: 23.9.93
Comments References: A 1.83kb *Eco*RI-*Hin*dIII fragment excised from the pgkNeopla in plC20 constructed by Brue Mann# K257 (originally from pGEM 7); fragment inserted into pBluescript SK(+) vector at *Eco*RI/*Hin*dIII



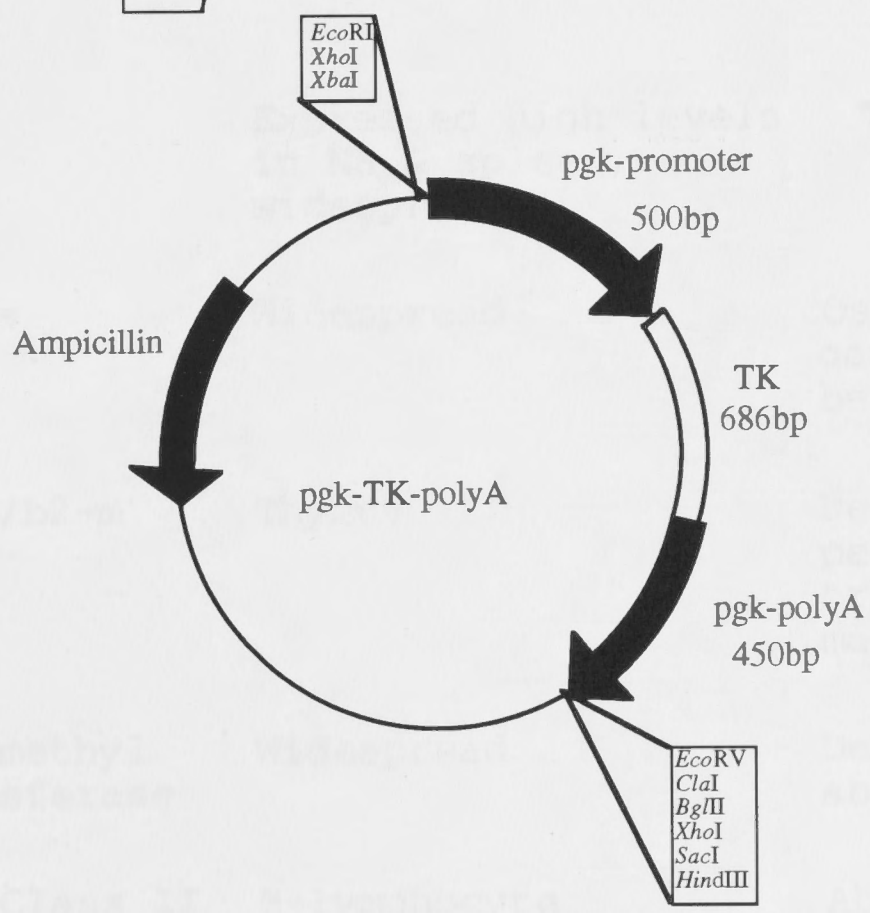
Plasmid Name: plC20-*Hin*dIII/*Eco*RI
Plasmid size: vector 2.67kbp/1.83kbp insert
Constructed by: Bruce Mann
Construction date: 1991
Comments References: Murine pgkpromoter driving Neo and pgkpolyA cloned into plC 20R

Appendix 2.4

pgkTKpla Cloning Vectors



Plasmid Name: pBSK(+)pgkTKpla
Plasmid size: 5600bp
Constructed by: X.-W. Wang & Dr K. Matthaei
Comments References: A 2.7kb *Eco*RI-*Hin*dIII fragment excised from the pgk-TK+pgk polA in pUC20 obtained from Brue Mann #258; inserted into pBluescript SK(+) vector at *Eco*RI/*Hin*dIII



Plasmid Name: pUC19pgkTKpla
Plasmid size: vector 2.67kbp/2.7kb insert
Constructed by: Bruce Mann
Construction date: 1991
Comments References: Murine pgkpromoter driving HSV-TK and pgkpolyA

Appendix 3.1 Gene Knockouts

(Reference: Shastry, 1994)

Disrupted gene	Tissue distribution	Phenotype
Prp protein	Neurons, lymphocytes & other tissues. Also expressed in tooth bud & placenta during development	Normal development & behaviour Resistant to Scrapie
Mouse Po	PNS	Hypomyelination, degeneration of myelin & axons
fyn	Widespread. Expressed high levels in brain, platelets & T cells	Hippocampal, T cell receptor signalling & LTP defects
lck	Present in T & B cells	Thymocyte differ. is blocked
csk	Expressed high levels in NS & spleen, widespread	Neural tube defects, embryonic lethality
c-fos	Widespread	Osteropetrossis,delayed gametogenesis, altered behaviour & lymphophenia
TAP1/b2-m	Thymus	Defects in intracellular peptide loading & or transport. Deficient in mature CD4 8*T Cells
DNA methyl transferase	Widespread	Death at mid gestation & abnormal development
MHC Class II associated variant chain	B-lymphocyte	Aberrant transp. of MHC ClassII molecules, deficient in producing CD4*T cells
Hoxb-4(hox-2.6)	Paraxial mesoderm/CNS	Anterior homeotic transformation
TNFR p55	Wide variety lymphoid & non-lymphoid cells	Normal thymocyte & lymphocyte population. TNF signalling & host response to specific stimuli are altered

Hox-1.1	Neural tube, spinal ganglia & sclerotomes	No abnormalities in growth property
TCR	Most systemic T cells	Loss of thymic medullae increase spleenic Bcells
Oct-2	B cell	No effect on edogenous immunoglobulin gene
Tyrosine Phosphatase	Hematopoietic cells & their precursors	B lymphocyte development is normal but T cell maturation is defective
Immunoglobulin μ chain	Pre-B cells precur. of B lymphocytes	B cell development arrested
Wnt-1 (int-1)	NS	Abnormalities in midbrain
GATA-1	Erythroid	Anemia
Insulin-like growth factorII	Many tissues choroid plexus in adults.	Growth deficiency
RNA pol II	All cells	No abnormalities
CD28	Peripheral T cells	Not required for T cell response
RB	Liver,Brain,Spinal cord and retina	Abnormalities of erythropoieses but not the retina
Myogenin	Muscle	Muscle defficiency & Neonatal death
RAR	Widespread	Postnatal lethality, testis degeneration
Zeta TCR	T cells	Influences thymocyte diff. but not required for T cell generation

Appendix 5.1 Examples of Gene Targeting Studies of Mammalian Behaviour

Targeted gene	ES	ES Origin	Chimaeras Crossed to	Genotype of Analysed Mice	Behavioral Change
En-2	D3	129	BL6,CD1,129	129-BL6,129-CD1	None reported
En-2	D3	129	129	Inbred 129	Impaired motor learning performance
5-htlB	na	129	129	Inbred 129	Enhanced aggresive behavior
fyn	na	129	129 & BL6	Inbred 129 & 129-BL6 hyb.	Impaired spatial learning
d2(dopamine receptor)	P1	129	BL6	BL6-129 hyb.	Decreased locomotory activities, impaired motor co-ordination
ε1 (NMDA receptor)	TT2	BL6xCBA	BL6	BL6-CBA hybrid	Impaired Spatial Learning
Glu recep δ2 locus	TT2	BL6xCBA	BL6	BL6-CBA hyb.	Decreased locomotory activities Impaired motor co-ordination
β2 (nACh-receptor subunit)	HMI	129	BL6xDBA/2	129-BL6-DBA/2hyb	Impaired motor avoidance learning
1-Adenylate	AB1	129	BL6	129-BL6 hyb.	Impaired spatial learning
mgluI	D3	129	BL6	129-BL6 hyb.	Motor impairment, impaired eye-blink condition

mglI	HM1	129	BL6	129-Bl6 hyb.	Decreased locomotory activities impaired spatial learning
NCAM	E14	129	not given	129-?	Impaired spatial learning
PKCγ	E14	129	BL6	129-BL6 hybrid	Impaired spatial & passive-avoidance learning
αCaMKII	E14	129	BALB/C	129-BALB/C hyb	Impaired spatial learning